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THE TEMPERATURE OF ACUTELY INFLAMED PERIPHERAL TISSUE.

By MARIO SEGÀLE.

(*From the Laboratory of Physiology of the University of Genoa, Genoa, Italy.*)

(Received for publication, August 22, 1918.)

INTRODUCTION.

The rise in the local temperature observed in peripheral areas of the body at the site of acute inflammation is generally attributed to the increased afflux of blood in these parts. This assertion is supported by the finding that in general the temperature of the inflamed peripheral tissue is not greater than the temperature of the internal parts and by the clinical observation that the temperature of the inflamed center is in proportion to the degree of the inflammatory hyperemia present. The fact is therefore excluded, and has recently been denied (1) that the inflamed center should have in reality a temperature higher than that of a corresponding area. The heat of the part has been attributed to the hyperemia.

The argument is of considerable theoretic importance and was discussed during the whole of the past century. The older treatises devoted many pages to the point, which appears now to have been solved as has already been stated. The subject has, however, scarcely received mention in the text-books, which as a rule paraphrase the statement of Cohnheim (2) made in his manual.

HISTORICAL.

The last known experiments on this point date from the year 1885. At that time Maximow demonstrated by a series of accurate determinations carried out with thermoelectric batteries that in no part of the inflammatory process is the temperature higher than that of the heart's blood or the blood of the larger vessels. Maximow thus confirmed what had been proved previously by Jacobson (3), Bernard (4), Schneider (5), and Huppert (6); namely, that if the inflamed center has a temperature higher than that of the corresponding area, it cannot be asserted that its temperature is above that of the central blood.

The older literature has a treatise by Weber which appealed to Liebermeister (7) who was conservative on this point, which Cohnheim (8) overlooked. Cohnheim merely stated that others more competent than himself had contradicted his statements.

Weber stated his experimental findings as follows: Measuring with thermoelectric batteries the temperature of the vein and that of the artery of the inflamed area, it is found that the venous blood is warmer than either the arterial blood from the same part or the venous blood from corresponding parts not inflamed.

The oblivion into which the controversy has fallen, particularly Weber's treatise, is demonstrated by the report of Galeotti (9), wherein Hunter, Weber, and Simon are mentioned as being of the same opinion, while in fact Hunter opposed the ideas of Weber and Simon.

The Problem.

I have failed to find that Weber's experiment was technically in error. It would seem to be a simple matter to establish whether the blood by its passage through an inflamed part gives off or absorbs heat. On the other hand, an impartial analysis of the problem leaves us uncertain as to the demonstrative value of the experiments mentioned in solving the point.

Considering the mass of tissue in the peripheral area in comparison with the volume of blood which circulates there, and bearing in mind also that the specific heat of blood is much like that of water, one is inclined to ask whether the blood is not a disturbing element, inasmuch as it conceals the real temperature of the inflamed center, giving to the part its own temperature in proportion to the blood volume which is renewed continually.

With regard to the liver, for instance, which has a much larger mass than a small inflamed area has, Claude Bernard proved that the circulating blood carries off heat from the parenchyma. Cavazzani measured an increase of over half a degree in the parenchyma when the hepatic artery and the portal vein were blocked. In cases of parotitis the classic experiments of Ludwig and Spiess (10) confirmed by Bayliss and Hell and Burton-Opitz (11) demonstrate an almost analogous fact: marked local heating and a temperature elevated above that of the arterial blood, with the venous blood warmer than the arterial blood.

It appears, therefore, that the experiment which I call Maximow's, although others have done it before him, cannot be considered exhaustive. On the other hand, our increased knowledge regarding the histology of the inflammatory process—and we know that there is an intense local metabolism—favors the idea that such metabolism may be accompanied by an increased release of heat, especially in view of the fact that most of the biologic reactions, as we know them, are strikingly disintegrating and isothermal in their effects. Virchow shared this view when he asserted the histogenetic origin and nature of the inflammatory process. Courmont (12) stated that to exclude all local production of heat contradicted accepted ideas.

Among the numerous authors who believe that the temperature of the inflamed area depends upon the increased afflux of blood, none has reflected on the physiological postulate that in general the tissues warm the blood and that it is not the blood which gives heat to the tissues. Claude Bernard (13) in discussing this point presented the problem as to whether the flow of blood to a part might not be considered the result of local calorification.

EXPERIMENTAL.

It seemed inadvisable to repeat Weber's experiment, because of the difficulties encountered in carrying it out accurately. It is necessary to be constantly near the animal under experimentation and also to make manipulations in the immediate vicinity of the thermoelectric batteries, which may readily lead to errors. Anyone who has had experience with this delicate instrument will acknowledge the difficulty of avoiding radiations of heat. Moreover, according to Weber's technique, it is necessary to introduce one electrode into the lumen of an artery and the other into that of an important vein. The possibility is always present that by this method the afflux and efflux of blood in the part may be altered in an indeterminable, irregular manner.

In a problem of this nature, with opinions so greatly at variance and so strongly impressed in the minds of pathologists, the services of expert technicians are required in order to eliminate criticism. In my experiments the difficulty has in the main been surmounted by regarding the blood not as the criterion, but rather as a disturbing element.

In the experiments presented here the attempt was made to compare the temperature of the inflamed tissue with that of the corresponding healthy area, at the moment when the circulation of blood was definitely arrested by the death of the animal or temporarily by ischemia. The temporary arrest of the circulation for thermostatic purposes is not a new device. Kussmaul and Tenner (14) tried it as far back as 1857 in the neuroparalytic hyperemia produced by incision of the sympathetic nerve in the area not inflamed. Cavazzani, as already stated, more recently used the same measure to establish the thermogenetic power of the liver.

The experience of other observers served as a guide in the choice of an indicator. Thermoelectric batteries, if connected to a sensitive measuring apparatus, are preferable to mercury thermometers which are slow, bulky, and require direct reading.[1]

Technique.

The electromotor force developed on the contact surface between two metals placed at different temperatures is indicated below. The electromotor force developed by the contact of two metals is equal to the difference between the values registered against them. For instance, the electromotor force developed between patent nickel and constantan is 8, between bismuth and antimony 100.

Electromotor Force Developed between Two Metals.

Bismuth	0
Constantan	30
Patent nickel	38
Nickel	51
Platinum rhodium	61
Platinum iridium	61
Platinum	66
Mercury	67
Gold, silver	74
Iron	83
Antimony	100

[1] I am indebted to Professor V. Grandis, Director of the Department of Physiology of the University of Genoa, for his courtesy in teaching me the technique and for his supervision of some of the experiments.

As in our experiments we were obliged to use thin wires, we could not employ bismuth-antimony electrodes, and on the other hand, not finding a sufficient quantity of constantan of the required diameter, Professor Grandis prepared from a disc of patent nickel (German nickeline) a long, thin wire which he soldered to a soft, iron wire as shown in the following diagram:

A and *B* were flattened and reduced to a lanceolated form. The highest possible potential was 45 microvolts for each degree of difference between 0 and 100. For measuring the developed potential we employed a Thompson galvanometer, to which the battery was attached directly after the internal resistance in respect to the inserted system had been regulated. After a number of attempts we succeeded in getting exact readings; *i.e.*, to 0.1 of a degree. This was done by adjusting the scale of the galvanometer. By means of this sensitive scale, the deviations did not require correction on direct reading, because the variations of temperature in the limits of observation were proportional to the angle of deviation of the galvanometer. This finding is corroborated by a number of observers.

To avoid abnormal radiations of heat which might lead to errors, the animal under experimentation was kept in a large, isolated room especially designed for calorimetric researches; here the temperature was practically constant. The readings of the valuations of potentiality were made in an adjoining room.

An inflammatory lesion was created by breaking, under ether anesthesia, the femur of a guinea pig without, however, injuring the skin. Inflamed areas appeared not earlier than 4 days later, or later than 12 days.

For the experiment one of the electrodes was introduced into the inflamed area of the guinea pig, while the other was inserted in a corresponding area on the other side. In general the introduction of the electrodes was not followed by considerable hemorrhage; when, however, hemorrhage did occur, the experiment was discontinued.

The system having been properly installed, the guinea pig was killed quickly by puncturing the medulla, or temporary ischemia of

the posterior part of the aorta was produced by compressing the thoracic portion. In the latter case, every precaution was taken to see that the heat from the compressing hand did not radiate to the electrodes. A bolometer was also adjusted to the animal, in order to record the reduction in temperature of the erythrocytes.

The protocols which follow do not give the absolute temperatures of the two parts, but rather the differences in temperature between the areas, as read directly from the galvanometer.

Experiment 1.—3 days after fracture of the left femur. At 2.40 p.m. one electrode was introduced into the inflamed area and the other into the corresponding area of the other limb.

Time.	Difference in temperature between inflamed and healthy areas.	Time.	Difference in temperature between inflamed and healthy areas.
p.m.	°C.	*p.m.*	°C.
2.48	0.8	4.45	3.0
2.49	0.9	4.55	3.0
2.55	0.8	5.10	2.6
3.05	0.7	6.15	1.9
3.18	0.9	6.30	1.7
(Killed by puncturing the medulla.)		6.40	1.7
3.21	1.3	6.50	1.6
3.24	0.9	7.05	1.4
3.30	1.0	7.15	1.4
3.37	1.9	7.30	1.3
3.45	2.3	9.35	0.9
3.53	2.7	10.05	0.6
4.00	3.0	10.35	0.5
4.10	3.1	10.55	0.5
4.18	3.2	11.00	0.5
4.40	3.1	After 10.00 a.m. the following day.	0.2

In the foregoing experiment the difference of the initial temperature of the inflamed area and the healthy area is seen to vacillate between 0.7° and 0.9°C. When the circulation of the blood is arrested because of the death of the guinea pig, the difference in temperature increases considerably. After 17 minutes it was 1.9°C. and after an hour 3.2°. This progressive derangement in the equilibrium of the post-mortem temperature of the two parts under the same conditions

may be considered as being due to slow cooling or to the persistence of a longer isothermal activity in the warmer area.

The hypothesis of retardation in cooling which first presents itself might be admitted as true if it were possible to demonstrate greater persistence of activity in the inflamed area, but this hypothesis cannot be readily advanced in this special case where the volumes and surfaces of the parts were small and equal in sensitivity. On the other hand, the hypothesis cannot be excluded, inasmuch as the surrounding temperature was low and the temperature of the body as a whole did not control the rate of cooling.

A reasonable hypothesis appears to be the assumption that the accentuation and maintenance of the differences of temperature stand in relation to differences of isothermal, biochemical activity of the two parts. In the inflamed area we have actively proliferating tissues. It is probable that the local life of the inflamed tissues continues for some time after circulatory and respiratory death has occurred, and that there is an emission of heat which differs according to the initial activities of the affected tissues. The difference in temperature remains high for $1\frac{1}{2}$ hours and then diminishes gradually, but it was still 0.5°C. after 8 hours.

But the fact which is particularly interesting for this work is the variation of the initial temperature and the increase of the difference between corresponding areas which takes place in a few minutes. This increase tends to confirm the principle first laid down; *i.e.*, that as the circulation of a volume of liquid at uniform temperature ceases, the local temperature becomes apparent. This in the case under consideration would be different; *i.e.*, higher in the inflamed zone than in the corresponding healthy area.

The following experiments elucidate these points.

Experiment 2.—Fracture of the left femur, 4 days before. Surrounding temperature 39.5°C. At 10.25 a.m. one electrode was introduced into the inflamed area and the other into the corresponding area of the other side. A bolometer was applied to the thorax.

Time.	Difference in temperature between inflamed and healthy areas.	Temperature of guinea pig.	Time.	Difference in temperature between inflamed and healthy areas.	Temperature of guinea pig.
a.m.	°C.	°C.	*p. m.*	°C.	°C.
10.35	1.2	39.0	12.10	3.2	27.8
10.40	1.2	39.0	12.17	3.0	
10.45	1.2	39.0	12.30	3.0	
10.47	(Killed.)		12.35	2.7	
10.49	2.2		1.30	2.0	
10.50	2.6	39.0	1.55	1.9	
10.51	2.6		2.25	1.5	
10.53	2.8	38.6	2.55	1.4	
10.55	3.0	38.6	3.15	1.2	
10.57	3.0	38.0	3.40	1.0	
11.00	3.1		4.20	0.9	
11.13	3.4	37.0	5.07	0.9	
11.32	3.4	33.4	5.50	0.8	
11.44	3.2		11.35	0.3	15.5
11.51	3.2				13.2

The experiment was conducted under the same conditions as those of Experiment 1.

During life the difference in temperature between the inflamed and the healthy zones was 1.2°C.; immediately after the death of the animal at 10.47 a.m. the difference increased. At 10.50 a.m., *i.e.* approximately 2 minutes after death, when the temperature of the bolometer was not sensibly altered, the local difference rose to 2.6°; *i.e.*, 1.4° more than during life. At 10.55 a.m., 5 minutes later, when the temperature of the body had fallen 0.4°, the difference between the two areas was 3°.

In this case we must exclude, I think, the idea that the causative factor is slower cooling, because the phenomenon manifests itself with a rapidity incompatible with that hypothesis. The temperature of the inflamed area was considerably higher than was apparent. The fact is demonstrated immediately when the disturbing influence of the blood which tends to carry off heat is no longer present.

The course of Experiment 2 corresponds approximately to that of the preceding experiment. The highest degree of difference was attained in a shorter period (after an hour, 3.4°C.), and it is noteworthy that even after 13 hours the difference was 0.3°.

In the preceding experiment we introduced a new element, the determination of the body temperature. It was shown that after a few minutes the general temperature had decreased 0.4°, while the difference between the local centers had increased 3°. This led me to consider it advisable to reduce the eventual causes of error consequent to external radiation. Experiment 3 was carried out in a room having a high and constant temperature (36° C.).

Experiment 3.—The guinea pig was placed in the calorimetric room which was kept at a temperature of 36°C. by means of electric lamps. A pair of batteries was connected with the fractured area and another pair with the corresponding healthy femur. The animal was provided with a bolometer.

Time.	Difference in temperature between inflamed and healthy areas.	Temperature of guinea pig.	Room temperature.
p.m.	°C.	°C.	°C.
2.20	3.1	39.4	36.0
2.25	3.1		36.0
2.27	3.1	39.4	36.0
	(Killed by puncturing the medulla.)		
2.31	4.6	39.1	36.0
2.40	4.6	39.0	36.0
2.47	3.6	39.0	36.0
2.50	3.0		
2.55	2.6		
3.03	2.1	39.0	35.8

In this manner, as the difference in the temperature of the guinea pig and that of the room was at a minimum, the curve of postmortem cooling should have had a slow course. From 2.28 p.m., when the animal was killed, until 2.30 p.m. the general temperature diminished 0.3°, and the difference between the local temperature of the inflamed area and the healthy zone increased 1.5°. A new fact is here brought out; *i.e.*, the markedly lessened permanence of the postmortem increase of thermal inequality between the two parts, the inflamed and the healthy areas.

This experiment does not give sufficient data to explain the fact,

but it should be noted that the initial difference in temperature of the inflamed center and the corresponding area was high, even before the guinea pig was killed, being about double the average. It is probable that the high room temperature which diminished the possibility of external radiation and kept the part warm, prevented the cooling effect of the blood. On the other hand, it is not excluded that the surrounding temperature itself may have caused some modification of the biochemical activity of the center by intensifying its activity.

To confirm and control the results that have been cited, other experiments were carried out. These consisted of experiments in which the circulation was interrupted permanently by the death of the animal and temporarily by local ischemia produced by compressing the abdominal aorta. In the latter case, it is possible to repeat the experiment a number of times and to modify it in details, thus by degrees neutralizing the errors of observation.

Experiment 4.—Fracture of the femur. The animal was placed in position at 1.30 p.m. Compression of the abdominal aorta at 3.10 p.m. and 3.21 p.m.

Time.	Difference in temperature between inflamed and healthy areas.	Time.	Difference in temperature between inflamed and healthy areas.
p.m.	°C.	*p.m.*	°C.
1.30	1.5	3.12	3.0
2.10	1.4	3.20	1.5
2.40	1.7		(Ischemia for 2 min.)
3.00	2.0	3.23	4.0
3.10	2.0	3.40	1.7
	(Ischemia for 2 min.)	3.50	1.5

The experiment could not be more convincing. Immediately following the ischemia the difference in temperature of the two parts became accentuated, but it returned to the initial value or somewhat below it as soon as the normal circulation of blood was restored. The experiment cannot, however, be considered perfect, because the determination of the differences was not made during the ischemia, but immediately afterwards.

Experiment 5.—Fracture of the femur 5 days before. Conditions the same as in other experiments.

Time.	Difference in temperature between inflamed and healthy areas.	Time.	Difference in temperature between inflamed and healthy areas.
a. m.	°C.	*p. m.*	°C.
10.10	1.8	2.20	1.2
10.30	1.7		(Beginning of ischemia.)
10.50	1.8	2.21	1.5
	(Beginning of ischemia.)	2.22	2.1
11.08	2.9	2.23	2.5
11.10	3.1	2.25	3.5
11.12	3.4		(Cessation of ischemia.)
	(Cessation of ischemia.)	2.27	1.2
11.14	1.9	2.45	1.2
11.20	1.8		

This experiment is a repetition of the preceding one, but marks an improvement in that readings were made during the ischemia; in addition, the second trial did not immediately follow the first, but was separated from it by an interval of 3 hours. An interesting point for further study is the fact that the initial difference in temperature of the inflamed and healthy areas amounts to 1.8°, while after 4 hours it is 1.2°. During the whole period when the thermoelectric battery remained in place, it probably initiated local injuries, and it is possible that these might have been the cause of an increased production of local heat.

The experiments reported above are supported and controlled by other analogous trials which are not given here because of the limitation of space. The experiments establish the fact that the difference in temperature of the inflamed and the corresponding healthy area increases rapidly and in a notable degree when the afflux of blood ceases because of a temporary stopping of the circulation or by a definite anemia. In view of these experiments made in an environment with a high temperature and taking into account for comparison the temperature of the body in relation to the time, we have excluded the possibility that the phenomenon depends upon retarded radiation of heat.

Because of the rapidity with which the phenomenon manifests itself, it does not seem probable that it could be related to a sudden

and more intense biochemical function of the inflamed cells stimulated to activity by an asphyxial condition.

It is consequently not the blood which heats the inflamed area, but, on the other hand, this area is kept by the active local hyperemia at a lower temperature than it would otherwise have. The finding which is apparently paradoxical is in fact true physiologically. In addition to the results cited by Cavazzani in his experiments on the liver, in muscle during contraction the venous blood is warmer than the arterial blood. The same is true of the parotid gland. Burton-Opitz (11) and Lefèvre speak of an equalizing temperature action of the circulation. In inflammation as well as in muscular contraction we have a notable local chemical activity. The circulation of the blood in both cases exercises its normal function, with the tendency of equalizing the temperature of the various areas.

The problem might be considered complete at this stage, but at the suggestion of Professor Grandis a more detailed study of the process was made, in order to establish if possible under what special conditions calorification develops and is influenced.

In the experiments made at a high room temperature it had been shown that the disturbance in thermal equilibrium of the corresponding areas was less permanent than at a low room temperature. Hence it seemed worth while to try the partial saturation of the organism with carbon dioxide to diminish the intake of oxygen into the tissues, or poisoning by cyanide of potassium or chloral to inhibit the local processes of oxidation. This series of studies, however, did not always lead to concrete results on account of the difficulty of attaining the required degree of saturation of the organism with the substances employed before death occurred. The experiments made with illuminating gas and cyanide of potassium failed to give convincing results, because death occurred at an early stage, when it was not probable that the toxic substances had been uniformly diffused in the organism so as to give the necessary degree of saturation. The experiments are therefore omitted. The results were better with chloral.

Experiment 6.—

Time.	Difference in temperature between inflamed and healthy areas.	Remarks.
a.m.—p.m.	°C.	
10.50	2.0	
11.20	1.8	
11.50	1.8	
12.30	1.8	
2.00	1.2	
	(Beginning of ischemia.)	
2.10	3.1	
2.15	1.4	
	(Cessation of ischemia.)	
2.20	1.5	
	(Chloral administered.)	
2.40	1.1	Animal alive.
2.45	1.2	" "
3.00	1.2	" "
3.20	1.0	Heart beating.
3.35	0.8	Seems dead.
3.40	0.8	Animal "
4.00	0.7	

This experiment confirms in the first place a diminution of difference in temperature of an inflamed and a sound limb after some hours of irritation of the healthy part. It also confirms the fact initially demonstrated, that the temporary ischemia always augments considerably and with great rapidity the disproportion of temperature of the parts. In addition, it establishes the fact that by administering a strong dose of chloral such as kills a guinea pig in a period varying from an hour to less than 80 minutes, there is an absence of the phenomenon normally observed when the guinea pig is killed by puncture of the medulla.

During the entire period in which the chloral manifested its toxic action and particularly in the interval between 3.20 p.m., when the guinea pig was certainly alive, and 3.40 p.m., when the animal was undoubtedly dead, the difference in temperature of the healthy and the inflamed areas did not increase; on the contrary, it showed a tendency to diminish. It is well known that chloral exercises an inhibiting effect on organic cellular oxidations (15). Even if my statements were not confirmed by the extensive literature on the subject,

which shows that there is a diminution of the carbon dioxide emitted and a lessened consumption of oxygen by the respiratory change, the suggestive experiments on the emission of heat before death, which diminishes with increased doses of chloral,[2] would be convincing.

These experiments, therefore, seem to indicate that the increase in the temperature of the inflamed area is due to a local cellular hyperfunction, for when this cellular activity is paralyzed the increase does not occur.

SUMMARY.

The experiments set forth here establish the fact that the heat of the inflamed part has its origin primarily in the local biochemical activity of the cellular elements which participate in the inflammatory process. The inflammatory hyperemia, instead of being the necessary and constant source of the inflammation must be considered a natural physiological compensation for the abnormal local calorification.

The rapid circulation of the blood in the inflamed part tends to moderate the increase in local temperature and to equalize the temperature with that of other parts of the body.

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A STUDY OF ATYPICAL TYPE II PNEUMOCOCCI.

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Study of the serological relation of strains of pneumococcus has shown that this organism may be classified by means of biological reactions into distinct types. From the first studies made it was evident that there existed three types of pneumococcus, the individual members of each type being alike in their immune reactions, the types themselves, however, differing sharply from one another. These types are now commonly known as Types I, II, and III. In addition to these fixed types there remain an indeterminate number of unrelated strains which for convenience have been grouped together and constitute the heterogeneous Type IV. More minute study of the last group indicates that it in turn can be further separated into a number of closely related immunological types.

The classification of pneumococcus was originally based upon immunological differences brought out by the reaction of protection. It later developed that the reaction of agglutination was as specific as the more time-consuming protection test, and recognition of this fact has led to its adoption as a routine method. In the course of the study of a large number of strains isolated from lobar pneumonia, it was observed that certain of these agglutinated atypically in Type II serum. Study of this phenomenon revealed the fact that these atypical Type II organisms possess partial antigenic characters common to the Type II pneumococcus, but vary from the typical representatives of this type by a diversity of relations among themselves, and by a lack of the reversibility of their immune reactions with the type organism. Because of these variations these organisms have been classified by Avery[1] as subvarieties of Pneumococcus Type II. In a study of ten such strains it was found possible to classify them

[1] Avery, O. T., *J. Exp. Med.*, 1915, xxii, 804.

into three distinct subtypes, IIa, IIb, and IIx. By means of agglutination, absorption, and protection experiments the members of Subtype IIa and IIb were found to possess immunity reactions identical with all other strains of the homologous types. Subtype IIx, however, consisted of a heterogeneous series of independent strains which showed neither cross-agglutination nor cross-protection.

The study reported in this paper was undertaken in order to determine the occurrence and frequency of atypical Type II pneumococci and their relation to lobar pneumonia, and to elaborate further their classification on the basis of their specific relations.

204 strains of pneumococci, all of which showed atypical agglutination in Type II serum, were studied. 77 strains were obtained from lobar pneumonia, 5 from postoperative pneumonia, 1 from meningitis, 5 from guinea pig pneumonia, 100 from normal mouths, 6 from convalescent Type I pneumonia, 3 from convalescent Type II pneumonia, 1 from convalescent Type III pneumonia, and 6 from dust.

In classifying these strains, agglutination and absorption reactions have been employed. In the agglutination reactions equal parts of broth cultures and immune sera were used, except where dilutions of sera were employed, when 0.1 cc. of culture in 0.9 cc. of immune serum diluted in normal salt solution was used. The agglutinations were incubated 2 hours in a water bath at 37°C., kept in the ice box over night, and readings made the next morning.

On the basis of specific agglutination in monovalent rabbit sera, the 204 strains were classified into twelve distinct groups as shown in Table I. From Table I it is seen that Subgroup IIb is the largest, representing 30 per cent of all strains studied. Next in order of frequency come Subgroups IIa, IIc, and IIm. These four groups comprise 73 per cent of all the strains classified. Subgroups IIa and IIb correspond to Avery's IIa and IIb, while the three strains originally placed in the IIx group were in the study of this series finally classifiable into Subgroups IIj, IIg, and IIe.

Only two strains showed cross-agglutination in the immune sera of a heterologous group. Strain St. 21 agglutinated promptly in serum of Subgroup IIm and slowly in that of Subgroup IIk. This culture was agglutinated by Serum IIm in a dilution of 1:160, but

only in a dilution of 1:80 by Serum IIk. A rabbit was immunized to Strain St. 21, and the type cultures, Nos. IIm and IIk, were tested for agglutinability in this serum.

From Table II it is seen that Culture IIm is agglutinated by Serum St. 21 in dilution of 1:300, while Culture IIk is affected by this serum only in a dilution of 1:100. Serum St. 21 was then exhausted

TABLE I.

Classification of 204 Strains of Type II Atypical Pneumococci.

Type.	Incidence.	
		per cent
IIa	38	19
IIb	62	30
IIc	28	14
IId	11	5
IIe	4	2
IIf	12	6
IIg	3	1
IIh	7	3
IIj	3	1
IIk	5	2
III	11	5
IIm	20	10

TABLE II.

Agglutination of Cultures IIk and IIm in St. 21 Serum.

Culture No.	Dilution of Serum St. 21.									
	1:10	1:20	1:40	1:80	1:100	1:160	1:200	1:240	1:300	Control.
IIk	++	++	++	+	+	−	−	−	−	−
IIm	++	++	++	++	++	++	++	++	++	−
St. 21	++	++	++	++	++	++	++	++	++	−

of agglutinins by absorption with Culture IIk, and its agglutinin titer determined for Cultures St. 21 and IIm.

From Table III it is seen that absorption of St. 21 sera with Culture IIk does not materially affect the agglutinins for Cultures St. 21 and IIm. Another lot of St. 21 sera was absorbed with Culture IIm. The results are shown in Table IV. From Table IV it is seen that

absorption of St. 21 serum with Culture IIm removes the agglutinins for both Strains St. 21 and IIk.

Strain P 65, which also showed cross-agglutination in heterologous sera, reacted slowly in Serum IIc and promptly in Serum IIm. This strain was tested like No. St. 21 by the absorption method and likewise showed that the reaction in Serum IIc was attributable to a minor agglutinin.

TABLE III.

Agglutination of Cultures St. 21 and IIm in St. 21 Serum Exhausted of Agglutinins for Strain IIk.

Culture No.	Dilution of Serum St. 21.									
	1 : 10	1 : 20	1 : 40	1 : 80	1 : 100	1 : 160	1 : 200	1 : 240	1 : 300	Control.
IIk	−	−	−	−	−	−	−	−	−	−
IIm	++	++	++	++	++	++	++	++	+	−
St. 21	++	++	++	++	++	++	++	+	+	−

TABLE IV.

Agglutination of Cultures St. 21 and IIk in St. 21 Serum Exhausted of Agglutinins for Strain IIm.

Culture No.	Dilution of Serum St. 21.									
	1 : 10	1 : 20	1 : 40	1 : 80	1 : 100	1 : 160	1 : 200	1 : 240	1 : 300	Control.
IIk	−	−	−	−	−	−	−	−	−	−
IIm	−	−	−	−	−	−	−	−	−	−
St. 21	−	−	−	−	−	−	−	−	−	−

Table V shows the source and type of the 204 strains studied. 77, or 38 per cent, were from cases of lobar pneumonia, 5 from post-operative pneumonia, and 1 from meningitis. 4 of the 5 strains from epidemic pneumonia in guinea pigs were isolated from guinea pigs in one city, while the 5th strain was from an epidemic in a different locality. All the guinea pig strains belonged to the same group. It has been shown that during convalescence from pneumonia the disease-producing types of pneumococci tend to disappear and are often replaced by Type IV or atypical Type II pneumococci, which have been previously shown to be the types most commonly found in

normal mouths. During convalescence the saliva of 6 Type I, 3 Type II, and 1 Type III patients showed atypical Type II pneumococci. If these are included, 110, or more than one-half of all the strains studied, may be considered as inhabitants of the normal mouth. 6 strains were recovered from dust.

From an examination of this table it is seen that the group may be divided according to the frequency of occurrence into two classes: (1) those found predominantly in disease; (2) those which occur more frequently in normal mouths.

TABLE V.

Source and Type of Atypical Type II Pneumococci.

Source.	Type.													
	a	b	c	d	e	f	g	h	j	k	l	m	Total.	
														per cent
Lobar pneumonia	33	12	2	5	1	2	1	7	2	2	6	4	77	38
Postoperative pneumonia	1	2			1							1	5	2
Meningitis												1	1	0.5
Guinea pig pneumonia			5										5	2
Normal mouths	2	38	18	6	2	10	2		1	2	5	14	100	49
Convalescent Type I	2	1	2							1			6	3
" " II		3											3	1
" " III			1										1	0.5
Total normal mouths	4	42	21	6	2	10	2	0	1	3	5	14	110	53
Dust		6											6	3
Total	38	62	28	11	4	12	3	7	3	5	11	20	204	

In the disease-producing class of atypical Type II organisms belong Subgroup IIa, with 33 strains out of a total of 38 from pneumonia, and Subgroup IIh, all of whose 7 strains are from pneumonia. Organisms of Subgroups IIb, IIc, IIf, and IIm are found frequently in healthy mouths, although they may also be found in association with disease. In some instances the groups comprise so few strains that it is impossible to determine their relation in either of these two classes.

During the last 3 years pneumococci have been isolated in 458 instances from 526 cases of lobar pneumonia admitted to the wards of

the Hospital of The Rockefeller Institute. These organisms have been typed according to the biological classification described by Dochez and Gillespie.[2] Of the 458 strains[3] obtained from disease, 52, or 11 per cent, were found to be atypical Type II organisms. Clough[4] in a study of 121 strains of pneumococci isolated from disease found 22, or 18 per cent, atypical Type II organisms. Sydenstricker and Sutton[5] in a study of 150 healthy individuals recovered atypical Type II pneumococci in 5 instances, and in 62 cases of pneumonia found this organism present in 12. They report a mortality

TABLE VI.

Type Incidence and Mortality of 59 Atypical Type II Pneumonias.

Type.	No. of cases of pneumonia.	Incidence.	No. of deaths.	Mortality.
		per cent		*per cent*
IIa	23	38	8	35
IIb	10	17	1	10
IIc	1	1	0	0
IId	5	8	3	60
IIe	1	1	0	0
IIf	1	1	0	0
IIg	0	0	0	0
IIh	7	12	4	57
IIj	2	3	0	0
IIk	1	1	0	0
IIl	5	8	1	20
IIm	3	5	1	33
Total.....	59		18	

of 31 per cent in pneumonia associated with atypical Type II pneumococci.

Table VI gives the mortality of 59 cases of lobar pneumonia from which atypical Type II pneumococci were isolated. 45 of these cases were in the Hospital of The Rockefeller Institute, 12 in the Presby-

[2] Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

[3] Stillman, E. G., *J. Exp. Med.*, 1917, xxvi, 519.

[4] Clough, M. C., *Bull. Johns Hopkins Hosp.*, 1917, xxviii, 306.

[5] Sydenstricker, V. P. W., and Sutton, A. C., *Bull. Johns Hopkins Hosp.*, 1917, xxviii, 312.

terian Hospital, and 2 were not hospital cases. Although these figures are too small to warrant any conclusion, the total mortality of 32 per cent indicates a high pathogenicity and virulence. The three subgroups, IIa, IId, and IIh, showed the highest mortality.

DISCUSSION.

Avery, working with only ten strains, was able to differentiate two definite subgroups, IIa and IIb. In the present study of 204 strains, ten other groups have been recognized. It is possible that if a larger series were studied, still other groups would be found. It is significant, however, that all of the 204 strains were classified in one or another of these twelve groups. Only two strains showed cross-agglutination in the immune sera of a heterologous group and these reactions were shown to be due to the presence of minor agglutinins for one group. Indeed it is surprising that the specificity of these groups is as definite as it is.

Although atypical Type II pneumococci are found associated with about 11 per cent of cases of lobar pneumonia, they have an incidence of 18.3 per cent in normal mouths. But as certain subgroups, IIa and IIh, are encountered more frequently in relation to disease, it is probable that they have a greater pathogenicity. These same groups occur rarely in normal mouths. Just the opposite is the case with Subgroups IIb, IIc, IIf, and IIm, which are much more frequently encountered living a saprophytic existence in normal mouths than in association with disease. Type I and II pneumococci cause a large percentage of pneumonia, but occur rarely in normal mouths. On the other hand, the Type III and IV organisms, although occasionally found in association with pneumonia, occur frequently in normal mouths. Similarly the atypical Type II pneumococci exhibit the same division into parasitic and saprophytic types as is already recognized in the major classification.

SUMMARY.

1. At least twelve subgroups of atypical Type II pneumococcus may be recognized by specific agglutination reactions. They have been designated Subgroups IIa, IIb, IIc, IId, IIe, IIf, IIg, IIh, IIj, IIk, IIl, and IIm.

2. These subgroups have an incidence of 11 per cent in lobar pneumonia, and of 18 per cent in normal mouths.

3. Certain groups, IIb, IIc, IIf, and IIm, occur in normal mouths.

4. Subgroups IIa and IIh are met with largely in connection with disease.

5. The mortality of acute lobar pneumonia due to these atypical Type II pneumococci is fairly high—32 per cent.

The author acknowledges his indebtedness to Miss Miriam P. Olmstead of the Presbyterian Hospital for 41 of the cultures of pneumococci used in this study.

THE OCCURRENCE OF BACILLUS INFLUENZÆ IN THROATS AND SALIVA.

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Plates 10 to 13.

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In the present paper are reported the facts obtained during an investigation of the bacteriology of influenza which included (1) the use of oleate hemoglobin agar, (2) the incidence of *Bacillus influenzæ* in uncomplicated and complicated cases of influenza and the associated types of pneumococci, (3) the occurrence of *Bacillus influenzæ* in convalescents, and (4) the occurrence of *Bacillus influenzæ* in the mouth secretions or throats of normal people.

The medium used in this work was Avery's oleate hemoglobin agar.[1] Agar of an average hydrogen ion concentration of 7.2 was used. As the domestic sodium oleate which was tested from time to time gave less consistent results, Kahlbaum's sodium oleate was used. This medium is especially favorable to the growth of Gram-negative organisms in influenza work. On these plates pneumococci do not grow and streptococci rarely grow, although some staphylococci show a scant growth (Figs. 1, 2, and 6). After 36 to 48 hours incubation several types of *Bacillus influenzæ* colonies are encountered on this medium. The characteristic colony of *Bacillus influenzæ* was clear, round, slightly glistening, convex, and discrete. The size varied from pin-point colonies, easily confused with diphtheroids or *catarrhalis* colonies, to large round colonies of 1 to 3 mm. in diameter. The larger characteristic colonies had a distinct nucleus which was much darker than the rest of the colony (Figs. 3, 4, and 7). The colonies were very sticky in consistency and would streak across the agar and follow the loop in long threads. The medium beneath the colony appears discolored after a colony is fished.

[1] Avery, O. T., *J. Am. Med. Assn.*, 1918, lxxi, 2050.

The colonies most readily confused on inspection with those of *Bacillus influenzæ* were those of *Micrococcus catarrhalis*, meningococcus, diphtheroids, and especially an unidentified organism called "Bacillus X." This organism was encountered in about two dozen individuals. The colony varied between 1 and 2 mm. in diameter, was clear, convex, and mucoid. The nucleus so common in the influenza colony was absent, but the colony left a slight discoloration on the agar. This bacillus is Gram-negative, takes the counterstain deeply, and when taken from agar surface cultures appears as long tangled threads, somewhat similar to the threads often seen in pure cultures of *Bacillus influenzæ*. In blood broth cultures it appears as a small fat bacillus without chain formation. It does not grow on plain sheep serum, or glucose agar. On blood agar the abundant growth appears as a clear, somewhat granular, fairly heavy film, with marked hemolysis of the red cells. This hemolysis is as marked as that produced by hemolytic streptococci (Fig. 5). Complete hemolysis is produced in blood broth after 24 hours incubation. After several days both blood broth and blood agar cultures turned brown and then blackish from the formation of methemoglobin. After 7 days incubation, litmus milk, to which a little blood had been added, showed an alkaline reaction with peptonization. No pathogenicity could be demonstrated for mice, rats, or rabbits.[2]

In all the cultures from convalescents and normal persons, and in many from the hospital patients, the organism was isolated in pure culture and was demonstrated to possess the typical morphology and shown to be strictly hemoglobinophilic. All evidence based simply

[2] The other organisms commonly found in cultures from the throat on this medium may be briefly described as follows in the frequency of their occurrence. (1) Gram-negative cocci of the *Micrococcus catarrhalis* group. The colonies are round, sharply defined, with a ridged or lined surface. Some colonies are so scaly that they lift off the medium as a pellicle; other colonies of this group will slide across the surface of the agar without breaking. (2) Staphylococci. Minute, pin-point colonies. (3) Diphtheroids. Minute, pin-point colonies. (4) Meningococci. The colonies are clear, round, and discrete. (5) Friedländer's bacillus. The colonies are white, mucoid, and occasionally confluent.

Occasionally in some plates a few Gram-positive streptococci may grow. These colonies are minute, dense, nucleated, and sharply defined. Pneumococci were never found and "spreaders" rarely grow.

on the presence of slender Gram-negative bacilli in films was discarded as of no value. The wide variations in the morphology of *Bacillus influenzæ* noted by Wollstein[3] were observed in this series. This study was made during the widespread epidemic of influenza which occurred in New York. The epidemic began about the middle of September, reached its height about the middle of October, and then gradually fell, though cases are still present in the city.

In Table I are shown the incidence of *Bacillus influenzæ* and the type of pneumococcus recovered from the patients admitted to the Hospital of The Rockefeller Institute, suffering from influenza,

TABLE I.

Incidence of B. influenzæ and Types of Pneumococci in Influenza and Pneumonia.

Disease.	No. of cases.	*B. influenzæ.*		Type pneumococcus.				No. of deaths.	Remarks.
				I	II	III	IV		
			per cent						
Uncomplicated influenza.	49	41	83+	0	2	2	17	0	
Influenza, with bronchopneumonia.	43	40	93	1	0	3	27	12	One case, Pneumococcus Types III and IV.
Bronchopneumonia.	6	6	100	0	0	1	4	0	
Lobar pneumonia.	20	11	55	3	2	6	7	7	One case, Friedländer's bacillus; one, staphylococcus.
Total.....	118	98	82	4	4	12	55	19	

bronchopneumonia, and lobar pneumonia during the 14 weeks between September 16, and December 31, 1918. In these cases of influenza and pneumonia the presence of *Bacillus influenzæ* was determined in the majority of instances by blood agar plates and mouse inoculation. The selective medium, oleate hemoglobin agar, was used only in the latter part of the investigation.

From 49 cases of uncomplicated influenza *Bacillus influenzæ* was recovered in 41 instances, or 83 per cent. From the 43 cases which were complicated by bronchopneumonia this organism was cultivated

[3] Wollstein, M., *J. Exp. Med.*, 1915, xxii, 445.

in 40, or 93 per cent. The 6 cases of bronchopneumonia, which were probably late cases of influenza, all showed influenza bacilli. The incidence of *Bacillus influenzæ* is much lower in the 20 cases of lobar pneumonia, as only 11 cases, or 55 per cent, were positive.

The distribution of the types of pneumococci in these cases is especially interesting. Whereas normally pneumococci of Types I and II are found associated with over 60 per cent of the cases of lobar pneumonia, in the cases of bronchopneumonia complicating influenza they are rare in comparison with Type III and IV pneumococci, which are normally and commonly found in the mouths of healthy persons.

In order to determine how many normal persons and those convalescent from influenza harbor influenza bacilli, a study was made of the personnel of the Laboratories and the Hospital of The Rockefeller Institute and the War Demonstration Hospital. From each individual a throat culture was taken with a sterile swab, which was then rubbed across a corner of the oleate hemoglobin plate. A small amount of mouth saliva was also collected in a sterile dish from each case. A loopful of saliva was later placed on a side of the plate. Each plate was streaked with a loop according to the Sunburst method[4] (Figs. 8 and 9). The plates were examined for *Bacillus influenzæ* after an incubation period of from 36 to 48 hours at 37°C.

From Table II it is seen that of 54 convalescent cases, 25, or 46 per cent, harbored *Bacillus influenzæ*. The length of time that had elapsed between recovering from influenza and the date of the culture varied from 1 week to 4 months. The shortest period in which a convalescent had apparently ceased to be a carrier of *Bacillus influenzæ* was 1 week. The longest period during which a convalescent was known to carry *Bacillus influenzæ* was 3 months. The average carrying period, as fixed by the time of taking the cultures, was about 6 weeks. Of 177 persons who gave no history of having had influenza, 74, or 42 per cent, carried *Bacillus influenzæ*. The total incidence of influenza carriers among the 231 normals and late convalescent individuals was 99, or 43 per cent.

[4] Standard technique for meningococcus carrier detection; adopted by the Medical Department of the U. S. Army, U. S. Navy, and U. S. Public Health Service.

In the above cases both throat and saliva cultures were made on oleate plates.

In all the 231 cases here represented, 73 showed a positive culture of *Bacillus influenzæ* from a throat swab (Table III). In each instance only a single throat culture or sputum culture was taken. 55

TABLE II.

Incidence of B. influenzæ in Convalescents and in Normal Individuals.

Individuals.	No.	Positive cases.	
			per cent
Convalescents	54	25	46
Normals	177	74	42
Total	231	99	43

TABLE III.

Comparison of Throat and Saliva Cultures.

Individuals.	Throat +; saliva −.	Throat −; saliva +.	Throat +; saliva +.	Throat −; saliva −.	Total.
Convalescents	14	6	5	29	54
Normals	30	20	24	103	177
Total	44	26	29	132	231

TABLE IV.

Incidence of Positive Carriers among Vaccinated and Non-Vaccinated Normal Individuals.

Vaccinated.		Non-vaccinated.		Vaccinated.		Non-vaccinated.	
Negative.	Positive.	Negative.	Positive.	Total.	Positive.	Total.	Positive.
					per cent		*per cent*
30	16	73	58	46	35	131	44

positive cultures of *Bacillus influenzæ* were obtained from saliva. This would seem to indicate that throat cultures are on the whole preferable to saliva cultures as a means of isolating *Bacillus influenzæ* from normal individuals or late convalescents. It must, however, be pointed out that in 26 instances *Bacillus influenzæ* was recovered from the saliva only, the throat cultures being negative.

Of the 177 normal people studied, 46 had received influenza vaccine. Of these, 35 per cent were positive carriers as compared with an incidence of 44 per cent among the 131 non-vaccinated individuals (Table IV). These observations were based on single cultures in each case. Thus it is apparent that there is no striking relation between vaccination and the carrier state. The importance of our observations is greatly lessened by the fact that many of those vaccinated had received their vaccine elsewhere and were as a rule unable to give any accurate information as to the dosage used.

DISCUSSION.

As a result of this study it is evident that during the influenzal epidemic period *Bacillus influenzæ* could be easily recovered from throats and saliva when a selective medium was used. The chief difficulty of isolating influenza bacilli from throats is analogous to that encountered in isolating meningococci from the nasopharynx; namely, that other organisms tend to overgrow *Bacillus influenzæ* just as they overgrow the delicate meningococci. The frequent occurrence of other Gram-negative bacilli, which morphologically may be easily confused with *Bacillus influenzæ*, renders the diagnosis of this organism from films alone valueless.

The high incidence of cultivation of *Bacillus influenzæ* from the upper respiratory tract of cases of influenza and bronchopneumonia during the epidemic is a point in favor of the view that this organism may be of significance in the disease in question. The fact that the incidence was high during this period in the cases of lobar pneumonia is not necessarily opposed to this view. Convalescent patients and normal individuals show about the same per cent of positive findings. It is noticeable that during the latter part of December, coincident with the ebb of the epidemic, the number of positive cultures decreased. The types of pneumococci found associated with influenza cases are of interest. Whereas normally 60 per cent of the cases of lobar pneumonia are associated with the presence of Type I and Type II pneumococci, these types are infrequently encountered in the pulmonary complications of influenza. On the contrary, the types which occur frequently in normal mouths, Types III and IV, are

usually recovered from the cases of bronchopneumonia which develop following influenza. At the same time a considerable number of the cases of lobar pneumonia occurring during this period were associated with the presence of pneumococci of Types I and II.

From this study it is clear that a diagnosis of *Bacillus influenzæ* is valueless if based only on the evidence obtained from direct films of saliva, throats, or cultures. It is also apparent that *Bacillus influenzæ* may be recovered in pure culture from a large percentage of acute cases of influenza, from convalescents, and from normal persons, if a suitable differential medium is used.

CONCLUSION.

1. Oleate hemoglobin agar is a good selective culture medium for *Bacillus influenzæ*.

2. *Bacillus influenzæ* has been cultivated from the mouth of 93 per cent of cases of influenza and bronchopneumonia.

3. *Bacillus influenzæ* was present at the time of this study in the mouths of 43 per cent of normal individuals.

4. The types of pneumococci found associated with the complicating bronchopneumonias of influenza are the types which are usually found in normal mouths.

EXPLANATION OF PLATES.

PLATE 10.

FIG. 1. Plain blood agar plate. The lateral sectors show growth of pneumococcus and *Streptococcus hæmolyticus*. The lower sector shows *B. influenzæ*; growth is present, but not visible in the photograph.

FIG. 2. Oleate hemoglobin agar plate. Corresponding sectors are planted with the same organisms as in Fig. 1 and show enhanced growth of *B. influenzæ* in the lower sector and complete inhibition of growth of pneumococcus and *Streptococcus hæmolyticus*.

PLATE 11.

FIG. 3. Pure culture of *B. influenzæ* on oleate hemoglobin agar showing large nucleated colonies after 36 hours incubation.

FIG. 4. Throat culture from a case of lobar pneumonia on oleate hemoglobin agar showing large nucleated colonies of *B. influenzæ* after 36 hours incubation.

FIG. 5. Blood agar plate showing hemolysis produced by hemolytic streptococcus (top) and that produced by "Bacillus X" (bottom).

PLATE 12.

FIG. 6. Growth of *B. influenzæ* on oleate hemoglobin agar; 15 hours growth at 37°C.

FIG. 7. Throat culture from a normal person on oleate hemoglobin agar showing large colonies of *B. influenzæ*; 36 hours growth at 37°C.

PLATE 13.

FIG. 8. Throat culture from a case of lobar pneumonia on oleate hemoglobin agar showing large colonies of *B. influenzæ*; 36 hours incubation at 37°C.

FIG. 9. Throat culture from a normal person on oleate hemoglobin agar showing many small colonies of *B. influenzæ;* 36 hours incubation at 37°C.

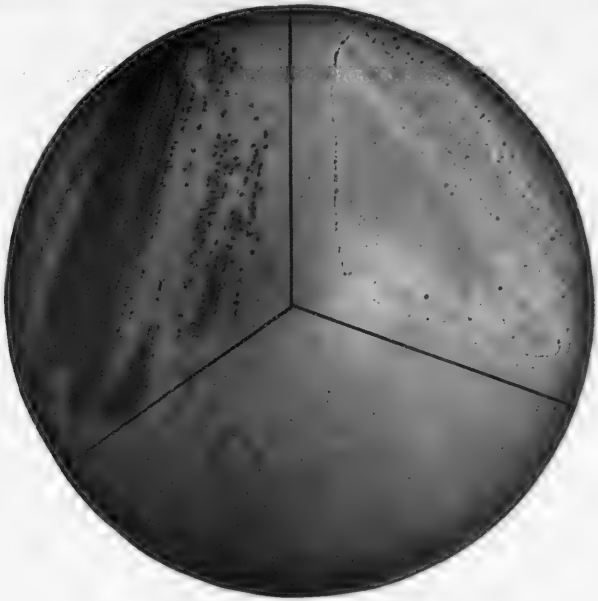

FIG. 1.

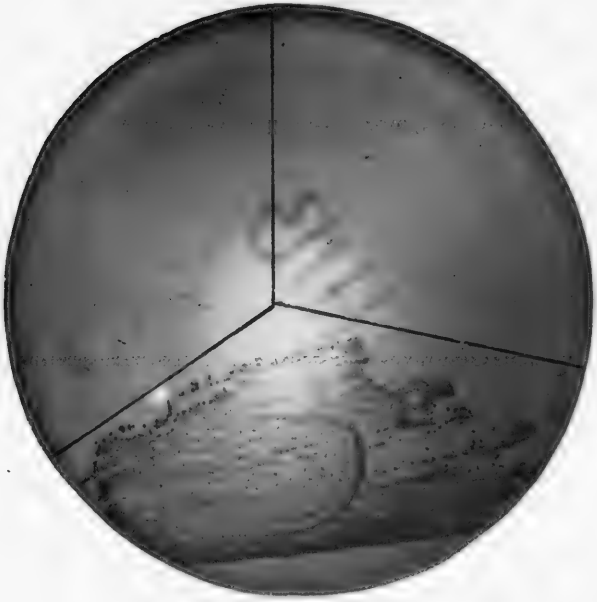

FIG. 2.

(Pritchett and Stillman: *B. influenzæ* in throats and saliva.)

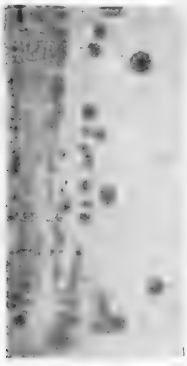

FIG. 3.

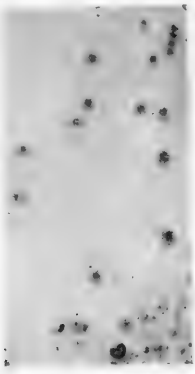

FIG. 4.

FIG. 5.

(Pritchett and Stillman: *B. influenzæ* in throats and saliva.)

FIG. 6.

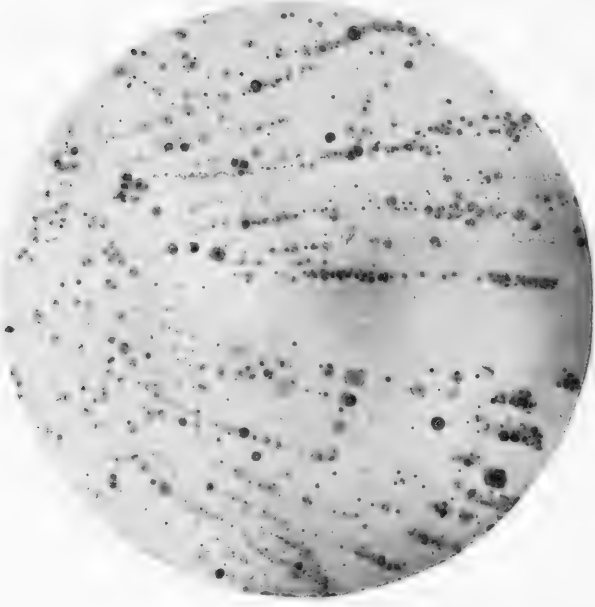

FIG. 7.

(Pritchett and Stillman: *B. influenzæ* in throats and saliva.)

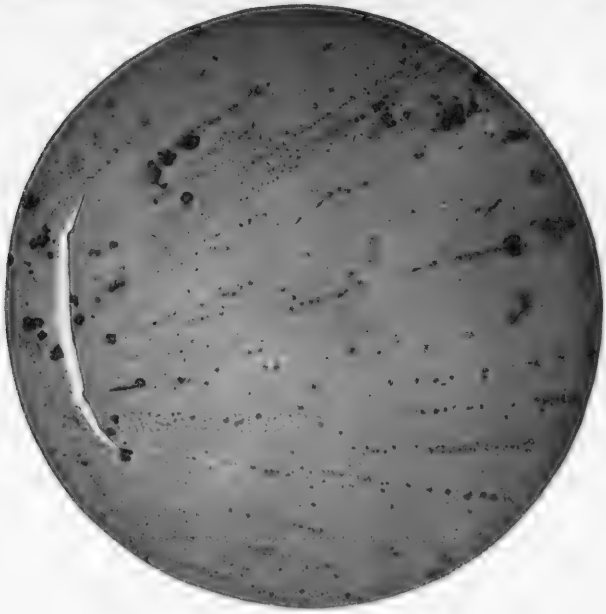

FIG. 8.

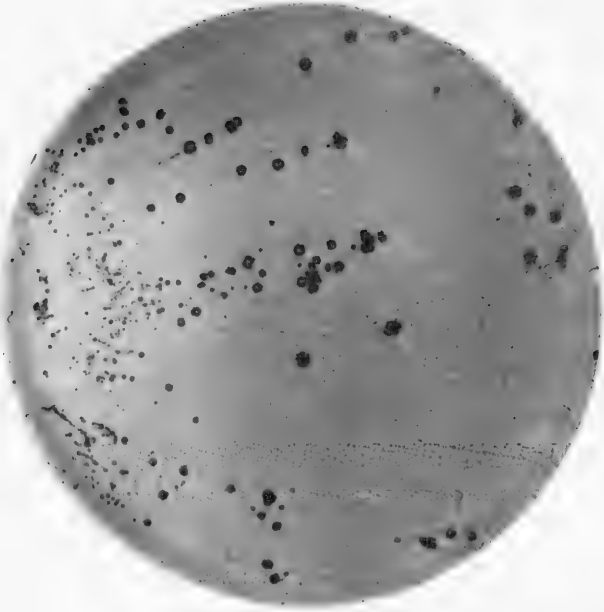

FIG. 9.

(Pritchett and Stillman: *B. influenzæ* in throats and saliva.)

THE DETERMINATION OF THE LENGTH OF LIFE OF TRANSFUSED BLOOD CORPUSCLES IN MAN.

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PLATE 14.

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Whether transfused blood corpuscles live and function for any considerable length of time, or whether the beneficial results that have been observed to follow transfusion, outside the purely mechanical part of increasing the bulk of the depleted blood, are due to a stimulating effect on the hemopoietic function by the product of the broken down corpuscles is still an open question (Archibald, Crile, Hunter, Kimpton, Primrose, Robertson and Watson).

Marfels and Moleschott (1856) and Brown-Sequard (1867) studied the question of the length of life of blood corpuscles by injecting nucleated corpuscles (bird and frog) into animals having non-nucleated corpuscles, and watching for the disappearance of the nucleated corpuscles from the circulation of the transfused animal. It is now known that these results have no bearing on the question, as it was not blood destruction that was being studied but the elimination of foreign protein. The same objection holds good for any attempt to stain blood corpuscles with so called vital stain and to reinject them, since there is no stain known that will hold without destroying the living quality of the corpuscles; therefore, in the stained corpuscle we are again dealing with a foreign body capable of more or less rapid removal than transfused blood.

Thus far the only satisfactory direct methods of attacking the problem of the length of life of transfused corpuscles have been those involving the study of the changes of the erythrocyte count following transfusion. Extensive work along this line, done on animals by Ward-Muller, Quinck, von Ott, and Hunter, was reviewed by Hunter in 1885 and 1886. Ward-Muller transfused dogs and produced

a plethora. 2 or 3 days afterward he found that the number of blood corpuscles corresponded closely to the number of the original corpuscles plus the transfused; a few days later the injected corpuscles, judged by the decrease in blood count, began to break down, and by the end of a few weeks all the injected corpuscles had disappeared. In order to produce the effect of a very slow transfusion Hunter injected blood intraperitoneally and found that the blood count increased up to the 2nd or 3rd day, after which it gradually decreased and reached normal from the 14th to the 26th day, when 40 to 90 per cent of the total blood volume was injected. Von Ott, in order to avoid the abnormal condition of plethora, removed from one-half to two-thirds of the blood from animals and replaced it with defibrinated blood. He noted a gradual fall in the blood count which reached a minimum from the 19th to the 22nd day. From then on an increase took place which reached normal 2 weeks later. The lowest part of this curve would not, of course, represent the last of the transfused blood corpuscles but the point after which their destruction was surpassed by the regeneration of new corpuscles. The time that elapsed before the complete disappearance of transfused blood was something more than from 19 to 22 days, a result which agrees well with the findings of the workers previously mentioned.

These and similar results, although they argue strongly in favor of the prolonged life of the red blood corpuscles after transfusion, have not been accepted as proof thereof, because they depend on the blood count uncontrolled by the volume of the blood. It is argued that the prolonged high blood count after transfusion may not represent an increased total number of corpuscles but an upset of long duration in the blood-volume-controlling factors, which results in a decrease in the fluid content of the blood and a consequent increased cell count.

Since the satisfactory determination of changes in the blood volume offers difficulties at present unsurmounted, it would seem that any method that could finally settle the question of whether or not the transfused blood corpuscles have any lasting existence in the blood stream must depend on some means of identifying the transfused corpuscle without in any way changing it by stains, etc. It seemed possible to do this in man by making use of the blood groups that are so well defined there. In man there are four blood groups, based on the ability of the serum of one group to agglutinate the corpuscles of another (Sanford). The corpuscles of Group I are agglutinated by the serum of all other groups but its serum has no agglutinating properties. The serums of Groups II and III mutually agglutinate each other's

corpuscles. Group IV serum agglutinates all other corpuscles, while Group IV corpuscles are not agglutinated by any serum. It follows then that a person in Group I may receive the blood of a person of any other group, and that the blood of a person in Group IV may be given to a person belonging in any of the other groups, since danger in transfusing blood of an unlike group arises only when there is agglutination of the incoming corpuscles, the transfused serum being too much diluted when mixed with the recipient's blood to produce any agglutination of his own corpuscles. Such transfusions, especially from the Group IV donor into the Group I, II, or III recipient, are fairly commonly done in the Mayo Clinic. I have found that when a blood whose corpuscles are agglutinable is treated with a serum capable of agglutinating them, the agglutination may, by using sufficient serum, be made practically complete. The count of the few corpuscles lying between the clumps (Fig. 1) is usually from 0.5 to 0.7 per cent of the total blood count, but it may vary in different bloods from 0.03 to 3.4 per cent. When, however, unagglutinable corpuscles are mixed with agglutinable corpuscles, either by transfusion or in the test-tube before the agglutinating serum is added, there is a very large number of free corpuscles present (Fig. 2) which appear in the proportion in which the two kinds of corpuscles are mixed. Since we have a means of separating the corpuscles of two groups that have been mixed by agglutinating the corpuscles of one group and leaving the corpuscles of the other unagglutinated, and since it is possible to transfuse a patient with a group other than his own, we may, by taking samples of his blood from time to time after transfusion and differentially agglutinating his own corpuscles, tell from the abnormal number of unagglutinated corpuscles present how long the transfused blood remains in the circulation.

Technique.

Preliminary experiments were made with mixtures of bloods to determine the factors that it was necessary to control in order to obtain a technique which would give uniform results. Mixtures of corpuscles of known count were made; these were treated with the agglutinating serum, and after varying amounts of shaking, incubat-

ing, etc., a count was made of the unagglutinated corpuscles in a red blood cell counting chamber. In this preliminary procedure a series of mixtures of blood to serum 1:1, 1:5.5, 1:11, 1:22, and 1:55 was made. Although the 1:1 mixtures gave numerous unagglutinated corpuscles, 1:5.5 did not give a consistently greater percentage than any of the mixtures in which a greater proportion of serum was used; therefore, the 1 : 22 mixture, which was adopted because of its adaptability to use in the white cell counting pipette, should secure a maximum agglutination. Shaking during incubation probably frees some of the unagglutinable corpuscles that might otherwise be caught in the clumps of agglutinated corpuscles and thus increases the count of unagglutinated corpuscles in mixtures in which the unagglutinable corpuscles are present. Allowing the blood to stand in the ice box over night after incubation appears to give more uniform results. Slight differences in the length of time in the ice box caused no practical changes. Allowing the tubes to become warm after being in the ice box caused a decided increase in the count; thus it is advisable to keep each tube in the ice box until the count is to be made. Tubes may be thoroughly shaken to produce an even suspension before making a count without producing any increase in the unagglutinated corpuscles. 10 minutes shaking in a mechanical shaker with glass beads in the tube increases the count of the unagglutinated corpuscles only 1 to 2 per cent of the total blood count, and here heat rather than the shaking is probably the factor that causes the increase. If the unagglutinated corpuscles are so thick that the count cannot readily be made, it is possible to make a dilution with cold serum. It is not advisable to use normal salt solution as that increases the count. Partly as a result of this preliminary work and partly for the sake of uniformity, the following technique was adopted.

Blood is taken from the ear in a white cell counting pipette to the 0.5 mark, the pipette is then filled to the 11 mark with the agglutinating serum to which a 4.4 per cent citrate solution has been mixed in the proportion of 20 : 1, and the whole is expelled into a small test-tube and shaken, thus giving a 1 : 22 mixture of blood and citrated serum. This mixture is incubated at 37°C. for 40 minutes with thorough shaking every 10 minutes and is left in the ice box over night. The mixture is then thoroughly shaken and a drop of it is placed in a

red blood cell counting chamber and a count of the unagglutinated corpuscles is made. 160 squares in each of two chambers are counted, the average is taken, and the count multiplied by $\frac{1,100}{2}$ to give the number of unagglutinated corpuscles per cubic millimeter of blood. A tube containing the blood of a person who has not been transfused is always used to control the effectiveness of the serum, and serum from the same person is used for agglutinating throughout a set of experiments.

Differential Agglutination in Vitro.

In vitro experiments were done to establish whether or not the results obtained by agglutinating the agglutinable corpuscles in a mixture of unagglutinable and agglutinable corpuscles were quantitative, and to determine whether, in such a mixture, the number of unagglutinable corpuscles counted is equal to the number actually present or whether it is some function of that number. To show the accuracy of the technique under absolutely uniform conditions with the same blood and serum and simultaneous shaking and incubation, two tests were made of each of a series of mixtures with different pipettes and the results compared (Table I).

These figures show a degree of accordance between each of two distinct tests which leaves no doubt as to their quantitative character.

In order to compare the number of unagglutinated corpuscles with the known number of agglutinable corpuscles present, mixtures of a Group IV blood with a Group II blood were made by the drop method in the proportions of 1:30, 1:15, 1:10, etc.; the same capillary pipettes were used throughout. Samples of these mixtures, which had been thoroughly shaken, were diluted in the white cell counting pipette with the Group IV serum, after which the usual technique was followed. Two agglutinations were made from each mixture and the count of the unagglutinated cells was averaged after subtracting the number of unagglutinated corpuscles found in the pure Group II blood, which in this instance was rather high, being 119,900 per c.mm. The number thus obtained was compared with the number of the Group IV corpuscles computed to be present, and found by dividing

the count of the Group IV blood used by the fraction which it composed of the whole mixture. The results are shown in Table II.

Through a wide range of mixtures the counted number of unagglutinated corpuscles approximated closely the number of unagglutinable Group IV corpuscles computed to be present.

TABLE I.

Proportion of Group IV corpuscles mixed with Group II corpuscles.	Red blood cell count of mixture.	Unagglutinated corpuscles in.		Tube 1 minus Tube 2.	Percentage of difference based on total blood count.
		Tube 1.	Tube 2.		
					per cent
1 : 30	4,880,000	235,400	209,000	26,400	0.58
1 : 25	4,870,000	249,700	228,800	10,900	0.22
1 : 20	4,840,000	335,500	325,600	9,900	0.20
1 : 15	4,800,000	474,200	464,200	6,600	0.14
1 : 10	4,710,000	578,600	566,500	12,100	0.26
1 : 5	4,500,000	894,300	887,700	6,600	0.15
Average percentage of error.....					0.25

TABLE II.

Proportion of Group IV corpuscles mixed with Group II corpuscles.	Unagglutinated corpuscles per c. mm. minus 119,900.	Calculated No. of Group IV corpuscles per c. mm.
1 : 30	127,370	137,000
1 : 15	228,570	267,000
1 : 10	391,600	388,100
1 : 6	572,773	610,000
1 : 4	803,559	854,000
1 : 3	1,035,320	1,067,500

Evidence that the Unagglutinated Corpuscles Observed after Transfusion Are the Transfused Corpuscles.

Before the abnormal increase of unagglutinated corpuscles found in the blood of Group I, II, or III patients transfused with Group IV blood could be assumed to be due to the presence of the transfused blood, it was considered necessary to run control experiments covering two points: (1) to see that this increase in count of unagglutinated

corpuscles does not appear when a patient with blood of an agglutinable group is transfused with blood of the same group, and, therefore, that the appearance of unagglutinated corpuscles is not some nonspecific reaction incident to the introduction of foreign blood; (2) to see that the appearance of the unagglutinable corpuscles is not due to a reaction of the transfused Group IV serum on the recipient's native corpuscles.

It was also of interest to note, in as far as it was practicable with our insufficient knowledge of the blood volume, whether there was any correlation between the amount of blood transfused and the number of unagglutinated corpuscles which appear in the circulation following transfusion with unagglutinable blood. That the increase in unagglutinable corpuscles does not appear except when unagglutinable blood is transfused is shown in the following results.

Of four patients who were transfused with blood of a like group, one gave 91,300 per c.mm. unagglutinated corpuscles before transfusion and 42,900 per c.mm. after transfusion, when 200 cc. of blood were transfused. In the three other cases, when 500 cc. were transfused, 51,700, 52,800, and 8,800 per c.mm. of unagglutinated corpuscles respectively were counted. As the agglutination with no transfusion has varied within the limits of from 0.03 to 3.4 per cent of the blood count, these figures are well within normal limits.

On the assumption that the appearance of unagglutinated corpuscles might be due to the transfused serum, the following experiment was done. During a transfusion on a Group II patient which lasted less than 30 minutes and in which 500 cc. of citrated Group IV blood were transfused, samples of blood were taken from the ear, one after 300 cc. and one after 500 cc. of blood had been given. A control had been taken immediately before the transfusion. The results obtained by agglutinating with Group IV serum were: before transfusion 41,690 unagglutinated corpuscles per c.mm., after transfusion of 300 cc. 269,000 unagglutinated corpuscles per c.mm., and after transfusion of 500 cc. 478,390 unagglutinated corpuscles per c.mm. Samples of blood taken before transfusion, both citrated and uncitrated, in this case and in others gave no increase in the unagglutinated corpuscle count when mixed with Group IV serum in the proportion of 1 : 22 and incubated for 24 hours. It would seem that if *in vitro* Group IV

serum does not affect the agglutinability of Group II corpuscles during 24 hours at body temperature in over 100 times the concentration in which it appears in the recipient's body, the immediate increase in unagglutinable corpuscles obtained during transfusion is not due to a reaction of any constituents of the transfused serum on the recipient's native corpuscles.

Whether or not the unagglutinated corpuscles appear in numbers proportional to the amount of blood transfused is shown in seven patients, of whom six were transfused with Group IV blood and one was a Group I patient who was transfused with Group III blood. The count of the unagglutinated corpuscles was compared with a computed figure obtained from the amount of blood transfused, the body weight, and a factor common to the series. If the correction is omitted which would be necessary if differences exist in different persons in the proportion between blood and body weight, concerning which unfortunately we have no satisfactory data, it may be assumed that the number of unagglutinated corpuscles which appear should be directly as the amount of blood transfused and inversely as the body weight. Taking x as the common unknown factor in Case A (Table III), in which the amount of blood transfused was 200 cc., the body weight 133.5, and the count of unagglutinated corpuscles 220,000, we have

$$220{,}000 = \frac{200}{133.5}\ x$$

Then by solving for x whose value is 146,850 and substituting in a similar equation in Case B in which the amount of blood transfused was 500 cc. and the body weight 151 pounds, a computed figure will be obtained, which may be called y, for the number of Group IV corpuscles which should be present per c.mm. Thus

$$y = \frac{500}{151} \times 146{,}850$$

$$y = 486{,}258$$

If we continue this process through the series, computed figures are obtained which give a basis of comparison of the results obtained in different cases. A comparison of these computed figures, with the

counted number of corpuscles, is given in Table III. If due allowance is made for the inherent factor of error in the computed figures it will be seen that the degree of agreement between these two sets of figures indicates a quantitative relation between the amount of blood transfused and the number of unagglutinated corpuscles which appear in the circulation after transfusion. The weight at the time of transfusion was used in all instances except in Case B who could not be weighed.

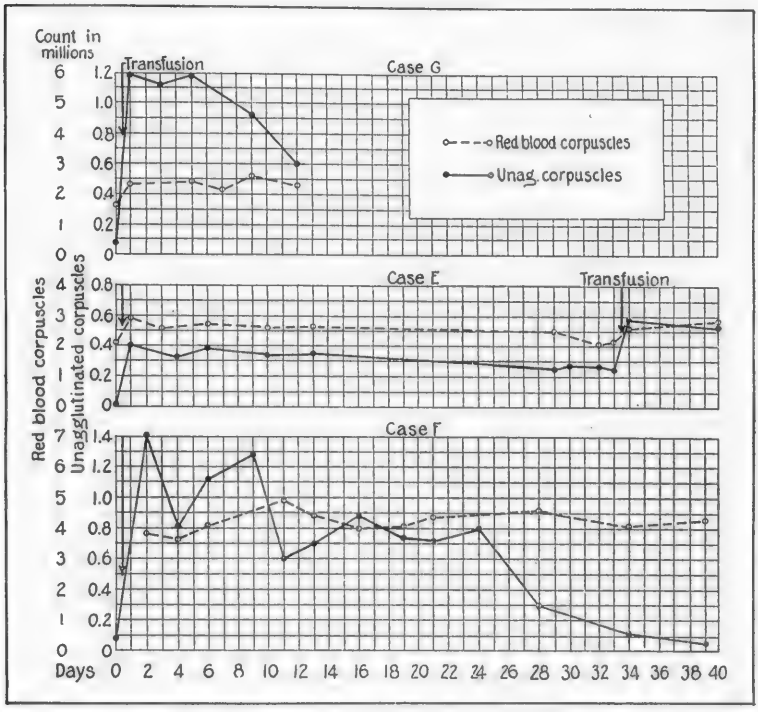
TABLE III.

Case No.	Reason for transfusion.	Weight.	Amount of blood transfused.	Calculated No. of Group IV corpuscles per c. mm.	Counted unagglutinated corpuscles per c. mm.
		lbs.	*cc.*		
A	Preparatory to operation.	133.5	200		220,000
B	Pernicious anemia. Transfused 20 days previously with 500 cc. of Group IV blood.	151 (normal).	500	486,258	551,000
C	Hemorrhage following Kraske operation.	96	500	764,843	698,000
D	Pernicious anemia. Transfused 19 days previously with Group IV blood. The initial count of unagglutinated corpuscles was 10 per cent of the blood count.	96	500	764,843	980,000 700,000 (minus initial count).
E	Anemia.	135	500	543,888	409,200
F	Hemorrhage following hysterectomy.	138	1,200	1,276,956	1,412,400
G	Anemia (infant).	20.5	250	1,790,853	1,175,900

Evidence of the Prolonged Existence of Transfused Blood Corpuscles.

Application of this technique was undertaken for the purpose of determining whether or not transfused blood lives in the circulation, and specimens of blood of patients of an agglutinable group transfused with Group IV blood were taken before, and at short intervals after transfusion. These specimens were examined for the persistence after transfusion of abnormal numbers of unagglutinated cor-

puscles. The protocols of Cases G, E, and F give the counts of unagglutinated corpuscles and a simultaneous study of the total blood count (Text-fig. 1). These figures represent the average of two pipettes. The unagglutinated corpuscles of the normal untransfused Group II individual who was used as a control varied through the experiments from 0.5 to 0.7 per cent of the total blood count.



TEXT-FIG. 1. The rate of change in the number of transfused corpuscles per cubic millimeter and the accompanying changes in the total blood count.

Case G.—Fat infant, age 17 months; weight 20.5 pounds. Belongs to Group II; had a low blood count. A diagnosis of secondary anemia had been made Sept. 20, 1918. The red blood cells were 1,660,000; the unagglutinated corpuscles were 54,400 per c.mm. A transfusion was done of 250 cc. of citrated Group IV blood.

Sept. 21. Red blood corpuscles 2,375,000; unagglutinated corpuscles 1,175,000 per c.mm.
" 23. " " " 1,107,700 " "
" 25. Red blood corpuscles 2,380,000; " " 1,192,400 " "
" 27. " " " 2,260,000.
" 29. " " " 2,570,000; unagglutinated corpuscles 933,900 per c.mm.
Oct. 2. " " " 2,280,000; " " 602,250 " "
" 4. Sent to the hospital with an attack of influenza.
" 7. Died.

Case E.—A Group II individual, age 32 years. Had a secondary type of anemia, possibly from hemorrhage due to piles. The patient's normal weight was 160 pounds, the present weight was 135 pounds. Oct. 1, 1918. The red blood cells were 2,100,000; the unagglutinated cells were 1,100 per c.mm.

A transfusion was done with 500 cc. of Group IV blood.

Oct. 12. Red blood corpuscles 2,820,000; unagglutinated corpuscles 409,200 per c.mm.
" 14. " " " 2,550,000.
" 15. Unagglutinated corpuscles 318,000 per c.mm.
" 17. Red blood corpuscles 2,705,000; " " 390,700 " "
Mild attack of influenza.
" 21. Red blood corpuscles 2,600,000; unagglutinated corpuscles 333,700 per c.mm.
" 24. " " " 2,660,000; " " 367,400 " "
" 25. Went home for 2 weeks.
Nov. 9. Red blood corpuscles 2,510,000; unagglutinated corpuscles 251,020 per c.mm.
" 10. " " 260,700 " "
" 12. Red blood corpuscles 2,105,000; " " 258,720 " "
" 13. " " " 2,185,000; " " 246,070 " "
A transfusion was done of 500 cc. of Group IV blood.
" 14. Red blood corpuscles 2,625,000; unagglutinated corpuscles 585,200 per c.mm.
" 20. " " " 2,760,000; " " 517,000 " "

Case F.—Age 35 years; normal weight 140 pounds; present weight 138. Operated on for fibroid of the uterus. Sept. 21, 1918. The unagglutinated cells were 88,000 per c.mm.; the blood count could not be obtained. 700 cc. of Group IV blood were given because of severe hemorrhage; the hemorrhage continued and an additional 500 cc. of Group IV were given.

Sept. 23. Red blood corpuscles 3,800,000; unagglutinated corpuscles 1,412,400 per c.mm.
" 25. " " " 3,720,000; " " 807,400 " "
" 27. " " " 4,090,000; " " 1,130,800 " "
" 30. " " 1,293,800 " "
Oct. 2. Red blood corpuscles 4,920,000; " " 590,700 " "
" 4. " " " 4,405,000; " " 704,000 " "
" 7. " " " 4,000,000; " " 870,100 " "
" 10. " " " 4,060,000; " " 737,000 " "
" 12. " " " 4,250,000; " " 702,900 " "
" 15. " " 796,400 " "
" 19. " " " 4,550,000; " " 299,200 " "
" 25. " " " 4,109,000; " " 118,800 " "
" 30. " " " 4,302,000; " " 51,700 " "

The greater irregularities in the count of unagglutinated corpuscles in this protocol are due to the fact that the patient's blood often clotted the Group IV serum with which it was mixed. This tendency was overcome later by increasing the strength of the citrate solution from 2 per cent to 4.4 per cent.

It will be noted in these cases that in each instance before transfusion there was an initial count of unagglutinated corpuscles which came within the limits of the counts found in other untransfused persons, while after transfusion there was a very great increase in unagglutinated corpuscles. In Case F, a patient who had had a severe hemorrhage and in whom the amount of blood transfused was great, this increase amounted to over 30 per cent of the total blood count; in Case G in whom the initial blood count was extremely low and the amount of blood given in proportion to the body weight was also great, it amounted to nearly 50 per cent of the patient's blood count. The increase was maintained for a long time with a surprisingly slow fall. In Case G who died 5 days after the last count was taken, only half the number of unagglutinated corpuscles which appeared after transfusion had disappeared in 12 days; in Case E whose condition remained on a level notwithstanding a slight attack of grippe, there was no marked decrease in the original number of unagglutinable corpuscles after 13 days, and in 33 days there was only a 37 per cent fall; in Case F, a comparatively normal patient who made an excellent recovery from operation, there was a marked decrease in unagglutinated corpuscles between the 25th and 28th days, and the agglutinability of the patient's blood may be considered to have approximately reached normal by the 30th day. In all cases the unagglutinated corpuscles, as long as they were present in considerable numbers, appeared to be in good condition.

I have shown that this differential agglutination reaction is a constant and quantitative one and that the unagglutinated corpuscles are present in numbers equal to the unagglutinable corpuscles admixed, so that unless something takes place in the body that does not take place in the test-tube, the counts of unagglutinated corpuscles after transfusion may be assumed to be a quantitative indicator of the number of transfused corpuscles present. As the unagglutinated corpuscles appear after transfusion in numbers proportionate to the amount of unagglutinable blood transfused, as they do not appear

when agglutinable blood is transfused, and, moreover, as they are not caused by any effect of the Group IV serum on the recipient's corpuscles, it seems safe to assume that these unagglutinated corpuscles are the transfused corpuscles and that their number is a quantitative indicator of the amount of transfused blood present in the recipient's circulation. It must follow then that the prolonged appearance of unagglutinated corpuscles in the blood stream of these transfused patients can only mean that the transfused corpuscles existed for a long time in the circulation, and it seems that they not only existed but functioned.

In considering these results, the question of to what extent they apply to the more usual condition when the patient is transfused with blood of his own group, immediately arises. Since the peculiarities which are brought out in blood grouping are probably peculiarities which will also be found in all the cells of the body, we may be able to turn to other tissues for light on this point. In this connection Masson's work in skin grafting is of interest.

"In all patients requiring skin grafting who were under my care during the past year, the blood of the donor, as well as that of the recipient, has been tested for agglutination. The results have been very interesting and instructive, and I feel sure will add a great deal to the popularity of the use of the isograft. . . . . I have tested the principle with the three varieties of grafts, and am satisfied that blood grouping is just as important for good results in skin grafting as it is necessary in transfusion, and that it is governed by the same principles. While the results are not positive, nevertheless, I have never had a skin take which was removed from a donor whose red blood corpuscles were agglutinated by the serum of the patient. The results in all other cases have been very satisfactory, almost, if not entirely, equal to autodermic grafting."

It would seem probable, if further investigation bears out the above results with skin grafts, that the results obtained in studying the length of life of transfused Group IV corpuscles in a recipient of another group hold for the life of the corpuscles of any group transfused into the recipient of a like group, and that it is even probable that these results hold for the length of life of the native corpuscle.

Since my results show that there is no immediate breaking down of transfused cells which could cause a stimulation of the bone marrow, and since it is more probable, as suggested by Lindeman, that when

there is a stimulation of the bone marrow following transfusion it is due to improved metabolic conditions of the bone marrow cells, brought about by the larger number of corpuscles in the blood, it would seem, on the surface, that the logical procedure in transfusion would be to push the transfusions until a normal blood count was attained, making the time interval between transfusions only long enough to allow the fluid content to establish itself. This would be indicated by the rise in the blood count which takes place a day or two after transfusion.

CONCLUSIONS.

1. It is possible in mixtures of corpuscles of different groups to separate the corpuscles practically quantitatively by treating with a serum that agglutinates the corpuscles of one kind, leaving the others unagglutinated.

2. After a recipient has been transfused with blood of a group other than his own, specimens of his blood treated with a serum that will agglutinate his own corpuscles but not the transfused corpuscles show unagglutinated corpuscles in large numbers.

3. These unagglutinated corpuscles which appear in the recipient's blood after such a transfusion are the transfused corpuscles and their count is a quantitative indicator of the amount of transfused blood still in the recipient's circulation.

4. The life of the transfused corpuscle is long; it has been found to extend for 30 days and more. The beneficial results of transfusion are without doubt not due primarily to a stimulating effect on the bone marrow, but, it is reasonable to assume, to the functioning of the transfused blood corpuscles.

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EXPLANATION OF PLATE 14.

FIG. 1. Microphotograph of the agglutinated blood of a person not transfused.

FIG. 2. Microphotograph showing the clumps of agglutinated native corpuscles and the unagglutinated transfused corpuscles of blood of an individual 16 days after transfusion.

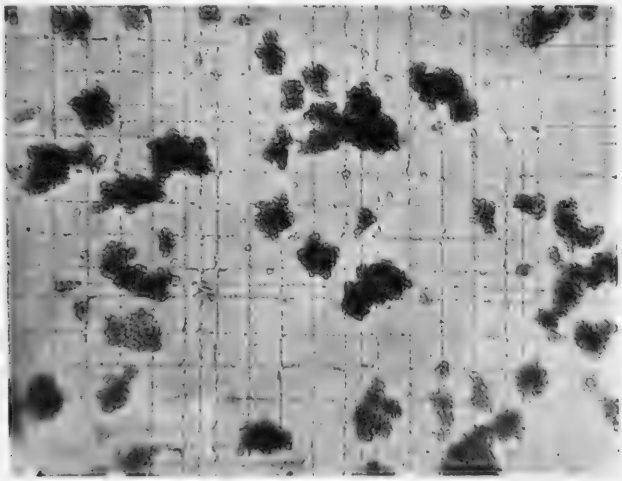

FIG. 1.

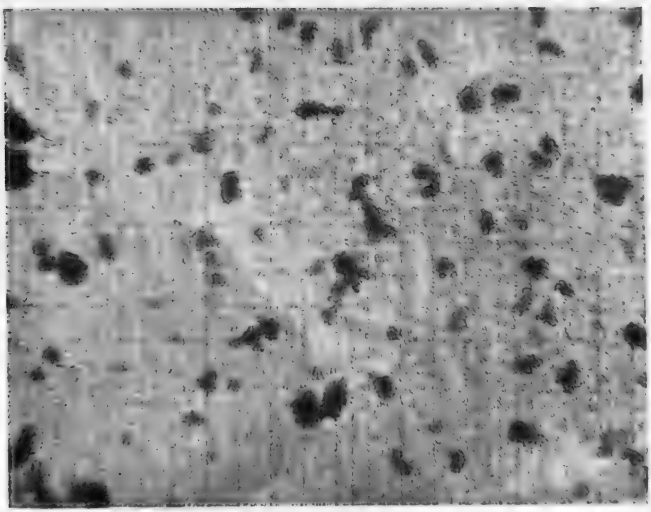

FIG. 2.

(Ashby: Transfused blood corpuscles.)

EXPERIMENTS ON THE PRODUCTION OF SPECIFIC ANTISERA FOR INFECTIONS OF UNKNOWN CAUSE.

I. Type Experiments with Known Antigens—a Bacterial Hemotoxin (Megatheriolysin), the Pneumococcus, and Poliomyelitic Virus.

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There are two generally recognized prerequisites to any attempt at the production in animals of an antiserum to combat an infectious disease. These are, needless to state, the isolation and the successful cultivation *in vitro* of the disease's causative agent. The difficulties here encountered in the case of some common and important maladies have absolutely held up all advances toward specific treatment.

The possibility of immunizing animals by direct injections of infected human tissues is one that must have occurred to many minds; and some attempts to employ the method were made prior to the general recognition that tissue itself acts to engender antibodies highly injurious to the species from which it is derived. With that recognition the method was given up, and it has not been revived. Yet there are noteworthy instances, such as the Pasteur treatment for rabies, which prove that tissue may contain an infectious agent in sufficient quantity to act as a practical antigen. And living tissues are superior in one respect to all other culture media, since organisms flourishing in them possess those pathogenic characters, loss of which in the test-tube sometimes limits, or indeed prevents, the development of antibodies in animals injected with cultures.

It has seemed to us possible that an antiserum produced by the injection of infected tissues might be rendered fit for employment in the species from which the tissues are derived by submitting it to a process of selective absorption, or exhaustion, with suspensions of

normal tissues. Such treated serum would, supposedly, be deprived of injurious tissue antibodies while retaining those directed against the infectious agent. A number of difficulties at once suggest themselves. It is well known that the absorption of antibodies is to a remarkable degree selective; and yet may not the repeated absorption of a polyvalent serum with tissue cells weaken notably the general content in antibodies? Will not toxic elements develop as a result of the incubation *in vitro* of mixtures of serum and tissue? And even should this not occur, is it really possible by the method of absorption to rid a serum of its anti-tissue potency so far as to render it harmless *in vivo*? May not the success of the absorptions be dependent on the employment of tissues derived from the same organ or organs used in the immunization? And finally, will not the method after all be more tedious than practical?

Some of these questions can be answered out of present knowledge. The recent work of many investigators on anaphylatoxins has shown that toxic elements are indeed engendered in incubated mixtures of tissue and its antibody, but only when complement is present.[1] In our selective absorptions inactivated serum could and should be used. The amount of tissue required to exhaust a serum of even high anti-tissue titer need not be great, since, as has been repeatedly shown in the typical case of red cells, many times the minimum hemolytic unit of amboceptor or agglutinin may be taken from a serum by a single unit of antigen. Furthermore, in the case of most sera, one can hope to use red corpuscles instead of tissues of the precise sort employed in the immunization. For much previous work has clearly demonstrated that only in certain special instances are specific cytotoxins the result of tissue injections.[2] Usually hemolysins, hemagglutinins, and serum precipitins alone develop.

In the work to be described our aim has been to determine the fundamental point, whether sera obtained by the immunization of animals with infected tissue of another species can, by the method of absorption, be rendered available for therapeutic use in the last mentioned species. No attempt has been made to devise practical

[1] See, for example, Friedberger, E., *Z. Immunitätsforsch.*, 1910, iv, 636.
[2] Pearce, R. M., *J. Med. Research*, 1904, xii, 1.

methods of absorption, to determine the least amount of tissue that will exhaust a serum, etc. For the purpose of type experiments sera have been selected containing different kinds of antibodies directed in all except one case—that of the chicken tumor—against pathogenic agents already isolated and well studied.

Selective Absorption Applied to an Antitoxic Serum.

The simplest case, that of an antitoxic serum, was first taken up. In view of the possible importance of "anaphylatoxins" engendered in the incubated mixture of serum and tissue as above mentioned, it seemed best to work with the animals most susceptible to such poisons; namely, guinea pigs. Furthermore, it was desirable to employ, if possible, a toxin of which the neutralization with antitoxin could be observed *in vitro* as well as *in vivo*. Both conditions are met by the hemolysin produced by *Bacillus megatherium* and first studied by Todd.[3] Todd demonstrated that the lysin is a true toxin, against which an antitoxin can be readily produced in guinea pigs, rabbits, and goats. His work has been fully confirmed.[4] *Bacillus megatherium* produces its toxin only when grown *in vitro* under special conditions, and although the toxin will kill guinea pigs, the organism itself is practically non-pathogenic. For this reason infected tissues could not be obtained for the immunizations required by our plan. Normal tissues mixed with the toxin might perhaps have been used, but the latter by itself, when injected locally, causes a violent inflammatory reaction. It was decided, on this account, to inject the toxin and the tissue used for immunization at separate sites and at different times.

Characters of the Megatheriolysin.—Three strains of *B. megatherium* were obtained from the American Museum of Natural History through the kindness of Professor Winslow. Only one produced any noteworthy hemolysin when cultivated in the special bouillon recommended by Todd.[3] This produced a lysin of such strength that 1 cc. of the Berkefeld filtrate of a 7 day culture, when in-

[3] Todd, C., *Lancet*, 1901, ii, 1663; *Tr. Path. Soc. London*, 1902, liii, 196.

[4] Dreyer, G., and Blake, J., *Lancet*, 1904, ii, 408. Craw, J. A., *Proc. Roy. Soc. London, Series B*, 1905, lxxvi, 179. Vincent, H., *Compt. rend. Soc. biol.*, 1909, lxvii, 195.

jected intravenously into a 400 gm. guinea pig, regularly caused intense hemolysis and hemoglobinuria, with death in a few hours at most. Intraperitoneally 2 cc. produced death quite as rapidly from diffuse petechial hemorrhages, first into the mucosa of the small intestine, and thence into the lumen of the intestine, which became distended with fluid blood. The uterus, large intestine, and stomach sometimes showed petechiæ, usually scattering. Despite the striking local lesions there was no evidence of intravascular hemolysis and never any hemoglobinuria, even when death occurred slowly. When given subcutaneously the lysin caused a wide area of necrosis.

The strain of *B. megatherium* furnishing the lysin was cultivated in quantity in Todd's medium, and at the end of 7 days the culture fluid was centrifuged and passed through a Berkefeld filter, after which the filtrate was tested for sterility, tubed, the tubes sealed with paraffin, and stored in the dark at 2–3°C. Under these conditions the lysin was found to retain practically all its activity for months, a great advantage, since portions of the same filtrate could be used both for the immunization of animals and for tests of the antisera that they yielded.

Immunization of Animals.

The lysin is most injurious to guinea pigs, yet large amounts are ill tolerated by rabbits or goats. The normal serum of both these animals has some slight neutralizing activity for the lysin, as shown by the ability to prevent hemolysis of guinea pig corpuscles. Attempts were made at first to immunize rabbits. A number of these animals were given six intraperitoneal injections at 6 day intervals of defibrinated guinea pig blood plus suspensions of ground liver and spleen, followed 2 days later by intraperitoneal injections of 1 cc. of the megatheriolysin. The animals were bled 7 days after the last lysin injection. They stood the immunization badly; all lost weight, and several died. Nevertheless, as will be seen, the survivors elaborated a well marked antitoxin.

A goat was immunized by separate subcutaneous injections of tissue and lysin according to the method just outlined. The finely ground liver, spleen, and kidney of guinea pigs, and the defibrinated blood were mixed and used. The amount of megatheriolysin given was gradually increased from 0.5 to 10 cc., diluted always with salt solution. This caused boggy swellings which were slow to subside. 7 days after the last of the six lysin injections the goat was bled, and the serum was tubed without the addition of preservatives and left in the ice box. The rabbit serum was similarly treated.

Method of Exhaustion with Red Cells.

The serum was inactivated at 56°C. for ½ hour just prior to use. Guinea pig red cells taken into citrate were thrice washed in ¼ per cent gelatin Locke's solution under aseptic conditions. In ordinary salt solutions guinea pig corpuscles

are prone to break down when washed; but the addition of a little gelatin to the washing fluid will prevent this.[5] After the last washing the cells were packed in graduated tubes, their bulk was noted, all possible fluid pipetted off, and the serum to be exhausted poured on, the tube corked, inverted, and gently shaken to suspend the cells. The suspension was now warmed to 38°C. in the water bath, incubated, centrifuged at high speed, and the serum transferred to a fresh lot of cells. The period of incubation varied with the degree of agglutination of the cells. When they fell out promptly into a solid mass such as could not be broken up without hemolysis, incubation for more than a few minutes was manifestly useless. But often after one or two absorptions the cells tended to remain in suspension, and the incubation period was lengthened to an hour. Finally, when the suspended cells showed no trace of agglutination, incubation was continued for as long as 2 hours in some instances.

No preservative was added to the mixture of serum and cells, but great care was taken to assure its sterility. The special corks for centrifuge tubes, elsewhere described,[5] were an aid in this connection. After the last absorption cultures were regularly taken. These showed an entire absence of infection, which may perhaps be attributed as much to the frequent centrifugation at high speed to which the serum was subjected as to our technique.

With repeated absorption there was a slight unavoidable loss of serum, and a slight dilution of it also occurred, owing to the remnant of gelatin Locke's solution introduced with the cells. In testing the relative potency of the unexhausted and exhausted serum usually no correction was made for this dilution of the latter.

Specimen Experiments—Rabbit Serum.

Experiment 1.

For this and all the subsequent tests the sera were inactivated at 56°C. for ½ hour. Whenever the period of incubation of the *in vitro* mixture is not specifically mentioned it was for 2 hours at 38°C. Readings were made after the tubes had stood over night in the ice box.

Hemolytic Activity of the Megatheriolysin.—This was determined as follows: 0.25 cc. of megatheriolysin in graded dilutions + 0.25 cc. of 0.9 per cent sodium chloride + 0.25 cc. of a 5 per cent suspension of washed guinea pig red cells.

Lysin strength*	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$
Hemolysis	C.	C.	C.	C.	+++	Ft. Tr.	0

* The lysin strength is expressed in terms of the undiluted material.

Antilytic Titer of Normal Serum.—The antilytic titer was tested of the inactivated sera of four normal rabbits, as contrasted with that of an animal repeatedly injected with megatheriolysin and guinea pig tissues. Mixtures were made of

[5] Rous, P., and Turner, J. R., *J. Exp. Med.*, 1916, xxiii, 219.

the sera in graded dilutions with a fixed amount of megatheriolysin, guinea pig red cells were added, and incubation was done. The amount of lysin in each tube was more than eight times that necessary under ordinary conditions for complete hemolysis of the corpuscles (*vide supra*). Subsidiary tests which need not here be given in detail showed that rabbit serum exercised its whole neutralizing effect on the lysin practically at once when mixed with it at room temperature. Consequently no interval was allowed to elapse before the red corpuscles were added.

0.25 cc. of rabbit serum in graded dilutions + 0.25 cc. of $\frac{1}{5}$ strength megatheriolysin + 0.25 cc. of 5 per cent guinea pig red cells.

Serum.		Serum dilution.							
		0	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Normal.	A.	++	+++	++++	Alm. C.	C.(?)	C.	C.	C.
	B.	+	+	++	+++	++++	Alm. C.	"	"
	C.	+	+	++	++	+++	" "	"	"
	D.	+	++	++	++++	++++	" "	"	"
Immune		0	0	0	Tr.	+++	" "	"	"

It is evident from this experiment that the normal rabbit sera possessed some power to prevent destruction of corpuscles by the megatheriolysin. But the immune serum conferred at least eight times as much protection, as shown by comparing its effect, when diluted, with that of the concentrated normal sera. The failure of the immune serum to protect to the same proportional degree in the higher dilutions is attributable to the presence in megatheriolysin of several lytic components[3] against all of which doubtless the antiserum had not the same relative activity.

Anti-Guinea Pig Titer of the Immune Serum.—This was well marked as a result of the repeated injection of the immunized rabbit with guinea pig tissue. A precipitin was present effective against dilutions of guinea pig serum up to and including 1: 256 when an equal bulk of the concentrated immune serum was mixed with it. Tests for hemolysis and agglutination were made as follows:

0.25 cc. of serum dilution + 0.25 cc. of 1 in 10 guinea pig complement + 0.25 cc. of 5 per cent guinea pig red cells.

	Serum dilution.											Complement + red cells + 0.25 cc. of salt solution.
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1,024	
Hemolysis	0	+	+++	++	±	±	Tr.	Tr.	Ft. Tr.	0	0	0
Agglutination	C.	C.	C.	C.	C.	C.	++++	±	Tr.	0	0	0

The low hemolytic titer was doubtless due in part to serum precipitation which took place in the mixtures, for complement is absorbed during such precipitation, as is well known. But it is also explained by the close biological relationship of rabbit and guinea pig which renders difficult the production of antibodies in the one against the other. The fact should also be recalled that specific hemolysins as a rule act but weakly when complemented with serum from the species furnishing the test cells. Yet it was deemed best to use such complement in our tests, since it would be the only one present during *in vivo* experiments.

Selective Absorption of the Anti-Guinea Pig Elements.—3 cc. each of the four normal sera and the immune serum above mentioned, all inactivated, were mixed respectively with 1.5 cc. of sedimented guinea pig red cells, incubated for 1 hour, and centrifuged, and the serum was transferred to a fresh portion of red cells. In the case of Normal Sera C and D only 0.5 and 0.65 cc. respectively of red cells were employed in the second absorption, while for the others 1.5 cc. were used as before. Incubation again was for 1 hour. In the first mixture of immune serum and cells a moderate agglutination was to be seen. None of the other mixtures ever showed the least trace of clumping.

Tests were made after the second absorption to determine how completely hemolysin and agglutinin had been removed from the immune serum. No trace of either was found. In view of these results tests to show whether the normal rabbit sera had been completely exhausted were deemed unnecessary, since such sera when untreated are almost devoid of antibodies for guinea pig red cells.

Antilytic Titer of the Treated Immune Serum.—0.25 cc. of immune serum in graded dilutions + 0.25 cc. of $\frac{1}{5}$ megatheriolysin + 0.25 cc. of 5 per cent guinea pig red cells.

Serum.	Serum dilution.							
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Untreated	0	0	0	Tr.	+++	Alm.C.	C.	C.
Exhausted	0	0	0	+++	Alm. C.	C.	"	"

The exhaustion with red cells had but little diminished the antimegatheriolytic titer of the serum.

In Vivo Tests of the Neutralization of Megatheriolysin with Exhausted Serum.—A number of mixtures containing 1 cc. of undiluted megatheriolysin with 0.75 cc. of serum or salt solution were incubated 2 hours, and 1.5 cc. of each were injected directly into the ear vein of a guinea pig. The method of injection has been described elsewhere.[6] The long period of incubation was unnecessary, since neutralization of the lysin is, as has been stated, practically instantaneous at room temperature.

[6] Rous, P., *J. Exp. Med.*, 1918, xxvii, 459.

Guinea Pig No.	Weight.	Mixture used for injection.	Result.
	gm.		
1	350	Exhausted immune serum + lysin.	Remained well.
2	350	Untreated " " + "	Died immediately after injection.
3	375	Exhausted normal serum (No. 1) + lysin.	Died 6 hrs. later; intense hemolysis; hemoglobinuria.
4	350	Exhausted normal serum (No. 2) + lysin.	Died 10 hrs. later; intense hemolysis; hemoglobinuria.
5	375	Exhausted normal serum (No. 3) + lysin.*	Died 40 hrs. later; intense hemolysis; hemoglobinuria.
6	375	Exhausted normal serum (No. 4) + lysin.	Died 10 hrs. later; intense hemolysis; hemoglobinuria.
7	350	Salt solution + lysin.	Died 13 min. later; intense hemolysis; at autopsy characteristic lesions.
8	350	" " + "	Died 12 hrs. later; intense hemolysis; hemoglobinuria.

* Part of injection material lost.

All the animals that succumbed, except No. 2, showed the lesions already described as characteristic of the megatheriolysin. In this case death was practically immediate, and there were no gross lesions except an almost complete intravascular hemagglutination, caused, of course, by the untreated immune serum, and without doubt the cause of death.

Experiment 2.

Another less comprehensive experiment with immune rabbit serum will be briefly quoted, since in addition to showing the antitoxic power of the exhausted serum it affords an interesting contrast between the lesions caused by the megatheriolysin, as such, and mixtures of the lysin with unexhausted serum.

The serum of an immunized rabbit was incubated as already described with two successive batches of washed guinea pig red cells. *In vitro* tests showed that the hemolysin and agglutinin were thus removed, whereas the antilysin was practically as strong as ever. The following mixtures were now made: (*a*) 2 cc. of megatheriolysin + 1.25 cc. of serum or salt solution; (*b*) 4 cc. of megatheriolysin + 2.35 cc. of serum or salt solution. After 2 hours incubation at 38°C. the whole of each mixture was injected into the peritoneal cavity of a guinea pig.

Guinea Pig No.	Weight.	Mixture (*a*). Lysin +	Result.
	gm.		
9	480	Exhausted immune serum.	Remained well; no anemia.
10	480	Untreated " "	Died after 48 hrs.; extreme anemia; hemoglobinuria.
11	480	Salt solution.	Died after 4½ hrs.; lesions characteristic of the megatheriolysin.
		Mixture (*b*). Lysin +	
12	500	Exhausted immune serum.	Remained well; no anemia.
13	500	Untreated " "	Died after 48 hrs.; progressive anemia; hemoglobinuria.
14	520	Salt solution.	Died after 1½ hrs.; lesions characteristic of the megatheriolysin.

The hemoglobin percentage in the blood of the surviving animals was followed for some days.

At autopsy the animals receiving megatheriolysin + salt solution presented the findings already described as characteristic after intraperitoneal injections. The small intestines were distended with blood from many fine hemorrhages into the mucosa. Other hemorrhages were present in the walls of the large intestine and stomach. The blood remaining in the vessels was unclotted, greatly concentrated, but unhemolyzed and unagglutinated. There was never any hemoglobinuria. The lesions from mixtures of the lysin with unexhausted serum were entirely different, being those characteristic of a serum hemolysin. A severe progressive anemia developed, accompanied by hemoglobinuria, and the blood specimens showed marked agglutination and many shadows. At autopsy there was no trace of intestinal hemorrhages such as result from the megatheriolysin. The spleen was greatly enlarged and crowded with phagocytes filled with red cells. There were also scattered ecchymoses on the pleuræ, diaphragm, and parietal peritoneum—a lesion never observed as the result of the megatheriolysin but commonly produced by a serum hemolysin. The conclusion is unavoidable that the animals had been saved from the action of the megatheriolysin only to succumb to that of the serum hemolysin.

Specimen Experiment—Goat Serum.

Experiment 3.

A freshly prepared lysin was employed for the work. Its titer diminished so little in the course of the 6 weeks during which a goat was repeatedly injected with it that tests made of the neutralizing activity of the serum of the animal before and after the immunization can be directly compared.

Hemolytic Activity of the Lysin.—0.2 cc. of megatheriolysin in graded dilutions + 0.2 cc. of 5 per cent guinea pig red cells.

Hemolysis.	Lysin strength.							
	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640
At first test	C.	C.	C.	C.	C.	Alm. C.	+++	Tr.
6 wks. later	"	"	"	"	Alm. C.	" "	+	"

Antilytic Titer of the Goat Serum before and after Immunization.—These tests were made at the same time as those of the megatheriolysin just quoted. 0.25 cc. of goat serum in graded dilution + 0.25 cc. of $\frac{1}{8}$ strength megatheriolysin + 0.25 cc. of 5 per cent guinea pig red blood cells.

Serum.	Serum dilution.									Whole strength serum + red cells + salt solution.
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	
Prior to immunization	+++	+++	++++	Alm. C.	Alm. C.	Alm. C.	C.	C.	C.	0
After immunization (6 wks. later)	0	0	0	±	C.	C.	"	"	"	
Serum of a normal goat (control)	Alm. C.	C. (?)	C.	C.	"	"	"	"	"	

The serum of the normal control was obtained and tested at the same time as the immune serum.

Plainly the immunization with megatheriolysin had increased the antilytic titer of the goat serum.

Anti-Guinea Pig Elements and Their Selective Absorption.—Preliminary tests showed that the serum of the immunized goat contained powerful antibodies for guinea pig red cells, as would naturally follow from the repeated injection of the animal with tissues of this species. An attempt was made to absorb the antibodies from a portion of the serum, and the titer of the exhausted specimen was then compared with that of an untreated portion. The normal control serum was not submitted to absorption because it was found to be harmless to guinea pigs when given in the doses required by our experiments.

For the purposes of exhaustion 17 cc. of the immune serum were incubated for 1 hour with 5 cc. of washed guinea pig red cells, the mixture was centrifuged, and

the serum transferred to more red cells and incubated again. This was done five successive times. In the first mixtures the red cells were only moderately agglutinated, but in the later ones clumping became much more marked owing doubtless to the absorption of proagglutinoids, which at first had hindered agglutination.

Mixture.	Hemagglutination.
17 cc. of serum + 5 cc. of red cells incubated 1 hr. and	Moderate.
serum transferred to 4.25 cc. of red cells, incubated 1 hr. and	"
" " " 7.6 " " " " " 1 " "	Strong.
" " " 7.3 " " " " " 1 " "	Almost massive.
" " " 5.2 " " " " " 1 "	Moderate.

Only 13.5 cc. of serum were finally recovered. The diminution in volume was due to a retention of the fluid amidst the agglutinated red cells. The latter, when clumped by the serum, occupied a greater space than when simply sedimented in salt solution.

A comparison was now made of the hemolytic and agglutinative titer of the exhausted and unexhausted immune serum, and of the normal control serum. 0.2 cc. of inactivated goat serum in graded dilutions + 0.2 cc. of 1 in 10 guinea pig complement + 0.2 cc. of 5 per cent guinea pig red cells were used.

	Serum dilution.												
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1,024	1/2,048	1/4,096
Serum.	Hemolysis.												
Untreated normal.....	+−	Tr.	Tr.	Ft. Tr.	0 (?)	0	0	0	0	0			
Untreated immune.....	Ft. Tr.	++−	++	+	Tr.	Ft. Tr.	Ft. Tr.	−	0	0			
Exhausted immune.....	No hemolysis.												
Serum.	Agglutination.												
Untreated normal.....	No agglutination.												
Untreated immune.....	+++	+	++	C.	C.	C.	C.	C.	C.	++	+	Tr.	0
Exhausted immune.....	+++	+++	+++	++	+	±	0	0	0	0			

Agglutination was read in the same mixtures as hemolysis. With rabbit complement the untreated immune serum was found to be far more hemolytic than with guinea pig complement as here shown. Precipitation was observed in the hemolytic mixtures containing immune serum in dilutions up to 1: 64, and to this is attributable the Neisser-Wechsberg phenomenon observed in the hemolytic tests of the untreated specimen.

Antilytic Titer of the Treated Immune Serum.—0.25 cc. of serum in graded dilutions + 0.25 cc. of $\frac{1}{8}$ megatheriolysin + 0.25 cc. of 5 per cent guinea pig red cells.

Serum.	Serum dilution.								
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Untreated immune	0	0	0	±	C.	C.	C.	C.	C.
Exhausted "	0	0	0	++	"	"	"	"	"
Untreated normal	Alm. C.	C. (?)	C.	C.	"	"	"	"	"

The repeated absorption of the immune serum with large amounts of red cells (29.35 cc. of the latter in all, as against only 17 cc. of serum) was found scarcely to affect the antimegatheriolytic titer, which remained more than eight times that of the normal serum.

In Vivo Tests of the Neutralization.

(*a*) *Intraperitoneal Injections.*—Mixtures of 2 cc. of megatheriolysin (or salt solution) with 2.5 cc. of serum (or salt solution) were incubated 1 hour at 38°C and injected into a number of guinea pigs of 400 gm. weight.

Guinea Pig No.	Mixture injected.	Result.
15	Normal serum + salt solution.	Remained well; no anemia.
16	0.9 per cent salt solution + lysin.	Died after 1¼ hrs. with characteristic lesions.
17	Normal serum + lysin.	" " 12 " " " "
18	Unabsorbed immune serum + lysin.	" " 53 " " progressive anemia and hemoglobinuria.
19	Absorbed immune serum + lysin.	Remained perfectly well save for a slight anemia soon repaired.

The order of injection was as follows: Nos. 15, 17, 19, 18, 16.

The findings at autopsy were similar to those of Experiment 2 with rabbit serum, as already described. All the animals that died, save No. 18, showed

lesions characteristic of the action of the megatheriolysin. In the guinea pig mentioned the lesions were of a different sort, being such as are caused by a specific hemolysin; and no trace of injury from the megatheriolysin was to be seen.

(*b*) *Intravenous Injections.*—Mixtures of 1 cc. of lysin (or salt solution) with 0.8 cc. of serum (or salt solution) were incubated 1 hour at 38°C., and 1.5 cc. of each mixture were injected into the ear vein of a guinea pig.

Guinea Pig No.	Mixture injected.	Result.
20	Normal serum + salt solution.	Remained well; no anemia.
21	" " + lysin.	Died after 42 hrs. with progressive anemia and hemoglobinuria.
22	Salt solution + "	Died after 47 hrs. with progressive anemia and hemoglobinuria.
23	Untreated immune serum + lysin.	Died after 8 hrs.; prostrated at once; hemoglobinuria.
24	Exhausted immune serum + lysin.	Remained well save for a moderate anemia.

Guinea Pig 20 weighed 425 gm., the others 450 gm.
The order of injection was as follows: Nos. 24, 23, 21, 20, 22.

Comment.

It is clear that by the method of selective absorption an antitoxic serum strong in tissue antibodies can be deprived of the latter to such extent as to be converted from a highly injurious agent into one capable of saving life. Indeed such a serum submitted to absorption five successive times, with a total bulk of corpuscles almost twice its own volume, was found to retain practically all its titer in antitoxin (Experiment 3).

A few of the guinea pigs receiving mixtures of exhausted serum and megatheriolysin were temporarily prostrated, lying on the side, and twitching, but they soon got to their feet and showed no permanent injury. The prostration occurred as frequently after the injection of normal serum as of that from animals immunized against guinea pig tissues. It is not possible to say whether the symptoms were due to "anaphylatoxin," or resulted from struggle under durance in connection with the rapid intravenous injection of a relatively large amount of foreign fluid. No such symptoms were ever observed after intraperitoneal inoculations.

The animals saved by the action of exhausted immune serum gave no evidence of injury to liver, spleen, or kidney, such as might perhaps have been expected in view of the fact that suspensions of these organs mixed with defibrinated blood were employed in the immunization. However, the only urine test made was for hemoglobin. Some of the guinea pigs receiving exhausted serum developed a slight or moderate anemia which was slow to appear and was most marked 4 or 5 days after the injection. Special experiments which need not here be cited in detail showed convincingly that the blood injury was due, not to unneutralized megatheriolysin, but solely to insufficient exhaustion of the serum with red cells, as proved by the persistence in it of hemagglutinins. When the absorptions were continued until all hemagglutinins had been removed, the serum was harmless to the blood. For example, it was found that the serum of Experiment 3 after seven absorptions no longer possessed hemagglutinins or produced anemia, whereas after only five absorptions it had both these characters as our protocol shows. The point is an important one, suggesting that the complete absence of hemagglutinins may be taken as the index to when exhaustion of a serum is complete. This indicator has been adopted, in much of our later work, and properly, as the results show. Hemagglutinins are far stronger than hemolysins in most sera resulting from prolonged immunization with animal tissues, and persist long after the latter have been removed by absorption.[7]

Selective Absorption Applied to an Antipneumococcus Serum.

The results of the work with an antitoxic serum were so encouraging that experiments were begun with sera of other types. It was highly desirable that they should be developed through the actual employment of infected tissues as antigen. The pneumococcus was selected for some of the tests, and attempts were made to immunize dogs against the organism by means of injections with the tissues of rabbits dying of pneumococcus septicemia. The difficulties encoun-

[7] An exception is to be noted in the case of anti-chicken sera from the goose and rabbit. These regularly contain hemolysins in fair quantity but only weak hemagglutinins.

tered illustrate strikingly the differences which may exist in the pathogenicity of a microorganism growing *in vivo* and *in vitro*.

Dogs possess a considerable resistance to the pneumococcus compared with some other species, as is well known; and they will often withstand the intravenous injection of several cubic centimeters of a bouillon culture fatal in minute quantity to mice. Intraperitoneal inoculations are even better borne. Nevertheless the immunization of dogs against the pneumococcus by intraperitoneal injections of blood or other tissues from rabbits moribund with pneumococcus septicemia proved well-nigh impossible, because of the high virulence of the antigen and our inability to standardize that derived from different rabbits. Small amounts of the infected tissue caused the dogs to die with a pneumococcus septicemia. Not infrequently they withstood a number of injections, only to succumb to one which was, quantitatively speaking, inconsiderable. In order to avoid this the infected rabbit tissue was heated *in vitro* at temperatures between 40° and 50°C. prior to injection, and the number of living organisms was thus reduced, as cultures showed, from millions to but a few per cubic centimeter. Still the injections often resulted fatally. Separate intraperitoneal inoculations of pneumococcus cultures and of normal tissues gave better results, but pneumococcus peritonitis so often ensued that at length separate subcutaneous inoculations were decided upon. These were carried out over a period of several months and a serum was finally obtained of sufficient antipneumococcus and anti-tissue titer to be suitable for experiments on selective absorption. We can confirm the observation of Nuttall[8] and Doerr and Moldovan[9] that antibodies of high titer are with difficulty elicited in the dog, as a response to immunization.

The plan of the experiments required yet a further alteration. Rabbits were found to vary so markedly in their resistance to the pneumococcus that very many would have been required had they been used as test animals in protection experiments with the antipneumococcus serum. It was decided, on this account, to exhaust the dog serum with rabbit red cells, in the manner that had proved successful with antimegatheriolytic serum and guinea pig cells, but to

[8] Nuttall, G. H. F., Blood immunity and blood relationship, Cambridge, 1904.
[9] Doerr, R., and Moldovan, J., *Z. Immunitätsforsch., Orig.*, 1910, vii, 223.

carry out protection tests with mice instead of rabbits. Mice were found to tolerate well the intraperitoneal injection of normal dog serum in the amount necessary for the work.

Immunization of Animals.—Nine dogs weighing from 8¼ to 13 kilos were injected intravenously with amounts varying from 0.5 to 1.25 cc. of the mixed citrated blood of three rabbits moribund with pneumococcus septicemia. The organism was of Type I (Neufeld strain), and the infected blood was preserved in the frozen condition for 4 days prior to use. Five of the dogs died of pneumococcus septicemia within a few days after the injection. The surviving four animals received injections of antigen at intervals of 7 days for more than 2 months. At first citrated septicemic blood was given intraperitoneally. This was badly tolerated, so recourse was had to inoculations with normal tissue and bouillon cultures of the pneumococcus, given at separate subcutaneous sites and on different days. The normal tissue consisted of suspensions of finely ground rabbit liver, spleen, and kidney mixed with defibrinated blood. Kidney tissue in special was employed, with the object of obtaining a serum that would be nephrotoxic. None of the dog sera acquired a demonstrable nephrotoxin, however, though hemolysins and hemagglutinins soon developed, and also weak agglutinins for the pneumococcus. The two most highly immunized dogs (Dogs A and B), as judged by these features, were bled for serum 9 days after the last pneumococcus injection.

Exhaustion of the Serum.—The method of selective absorption was that already described in connection with antimegatheriolytic serum. Rabbit red cells, thrice washed, were packed by rapid centrifugation, all possible fluid was removed, and the cells were mixed and incubated with the serum under test. The latter was in this way exhausted by contact with several successive portions of cells.

Experiment.—The sera of two immunized dogs and three normal controls were inactivated at 56°C. for ½ hour, and portions of all were submitted to an exactly similar exhaustion with red corpuscles. The incubation period was 1 hour with each successive batch of cells.

Mixture.	Hemagglutination.		
	Immune sera.		Normal sera.
	A	B	
26 cc. of serum + 3⅓ cc. of red cells, incubated 1 hr. and	Massive.	Heavy.	
serum transferred to 3⅓ cc. of red cells; incubated 1 hr.	Moderate.	Moderate.	
" " " 4 " " " " " 1 "	Slight.	Slight.	0
" " " 4 " " " " " 1 "	Tr.	Ft. Tr.	

Cultures taken after the last absorption showed all the sera to be sterile.

Anti-Rabbit Titer of the Immune Sera. Hemolysis.—0.2 cc. of inactivated serum in graded dilutions + 0.2 cc. of 1 in 10 guinea pig complement + 0.2 cc. of 5 per cent red cells.

Serum.		Serum dilution.										
		0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1,024
Dog A	Untreated....	C.	C.	+++	++	++−	+	+	Tr.	Ft. Tr.	0	0
	Exhausted....	No hemolysis.										
Dog B	Untreated....	C. (?)	Alm. C.	Alm. C.	++++	++	+−	+−	Tr.	Ft. Tr.	0	0
	Exhausted....	No hemolysis.										

Hemagglutination.—The mixtures were the same as those for hemolysis save that 0.2 cc. of salt solution was substituted for guinea pig complement.

Serum.		Serum dilution.										
		0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1,024
Dog A	Untreated..	C.	C.	C.	C.	+++	+++	++	Tr.	Tr.	0	0
	Exhausted..	++++	+++	++	Tr. (?)	0	0					
Dog B	Untreated..	C.	C.	Alm. C.	++++	++++	++	Tr.	0	0	0	0
	Exhausted..	++	Tr.	0	0							

Although some hemagglutination was noted in the mixtures with exhausted serum after they had stood over night, no trace of this was observable when they were first taken from the incubator. Many hemagglutinins, as Landsteiner and Reich[10] first showed, act most strongly at low temperature.

Because of these findings, which showed that the exhaustion of the immune sera with rabbit cells had been practically complete, no similar tests were made of the exhausted normal sera. For the anti-rabbit titer of these sera, when unexhausted, was, relatively speaking, slight.

Pneumococcus Agglutinins.—These were present in the immune sera but were weak, being effective on macroscopic test only in dilutions up to 1 in 16 of each serum. They were found to be unaffected by exhaustion of the sera. The details of the tests need not be given.

In Vivo Tests of the Protective Power of the Exhausted Antipneumococcus Serum.—With the change in plan that determined the use of mice instead of

[10] Landsteiner, K., and Reich, M., *Centr. Bakteriol., 1te Abt., Orig.*, 1905, xxxix, 83.

rabbits for the protection experiments, it became unnecessary to make *in vivo* tests as to whether the sera exhausted with red cells had been deprived of their toxicity for the species furnishing the cells; that is to say, for rabbits. The results with exhausted anti-guinea pig serum of far higher original titer, which have already been described, were deemed sufficient on the point, especially since tests showed that the unexhausted dog serum contained not the least nephrotoxin for the rabbit, despite the repeated use of the renal tissue of rabbits as an antigen. The method adopted for the protection experiments with mice was that now familiar from the work of Avery, Chickering, Cole, and Dochez.[11] An 18 hour culture in pneumococcus broth of the Neufeld strain of pneumococcus was used in a series of tenfold dilutions with this broth. Of each culture dilution 0.5 cc. was drawn into a Record syringe, then 0.5 cc. of the serum under test was taken up, and the whole was at once injected into the peritoneal cavity of a 20 gm. mouse. The control sera were handled after the immune sera, so that the former had the advantage of any attenuation in the pneumococcus suspensions which might have occurred during the period of the injections. Last of all, some mice were given a set of control mixtures containing 0.5 cc. of broth instead of a serum.

The animals that died were autopsied promptly and films taken of the peritoneal exudate and heart's blood. These in every case showed the pneumococcus in pure culture. No mouse was put down as surviving until 4 days after injection.

Serum.		Amount of culture.					
		0.000001 cc.	0.00001 cc.	0.0001 cc.	0.001 cc.	0.01 cc.	0.1 cc.
		hrs.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
Broth controls		D.* in 26	D. in 28	D. in 32	D. in 20	D. in 16	
Immune A	Unexhausted	Lived.	Lived.	" " 42	" " 26	" " 23	D. in 17
	Exhausted	"	"	Lived.	" " 89	Lived.†	" " 11
Immune B	Unexhausted	"	"	"	Lived.	D. in 17	" " 14
	Exhausted	"	"	"	D. in 32	" " 49	Lived.
Normal a	Unexhausted	D. in 30	D. in 30	D. in 23	" " 17	" " 17	D. in 7½
	Exhausted	" " 31	" " 21	" " 20	" " 16	" " 11	" " 9
Normal b	Unexhausted	" " 27	" " 30	" " 25	" " 22	" " 12	" " 9½
	Exhausted	" " 31	" " 50	" " 22	" " 11	" " 9	" " 9
Normal c	Unexhausted	" " 30	" " 42	" " 19	" " 14	" " 14	" " 7½
	Exhausted	" " 40	" " 28	" " 28	" " 22	" " 12	" " 10

* D. indicates died.

† Slight escape of injected fluid beneath skin.

[11] Avery, O. T., Chickering, H. T., Cole, R., and Dochez, A. R., Acute lobar pneumonia. Prevention and serum treatment, Monograph of The Rockefeller Institute for Medical Research, No. 7, New York, 1917.

Comment.

The experiment shows that the immune dog sera protected mice against about 100 times the amount of pneumococci that was fatal when normal dog serum was employed. The exhaustion of the immune sera with four successive portions of rabbit red cells did not diminish in the least its protective character. The question may be asked, why was such an oblique method used to demonstrate the availability of the exhausted serum? Instead of immunizing dogs with rabbit tissue, exhausting the dog serum with rabbit red cells, and testing protection on mice, might not these latter animals have been employed throughout, to the elimination of rabbits? This was not practicable for several reasons. It would have been difficult to obtain enough sterile normal tissue from mice for the production of a strong anti-mouse serum, and, granting that such a serum could eventually have been elicited, the problem would have arisen of obtaining sufficient mouse red cells for its exhaustion. Undoubtedly the spleens of mice dying of pneumococcus infection would have furnished a powerful antigen, as concerns this organism; but so little of the splenic tissue could have been employed in the immunization, owing to the virulence of the pneumococci therein contained, that it is doubtful whether the serum resulting would have been strongly anti-mouse. And a serum strong in anti-tissue elements was desirable for our type experiments.

Selective Absorption Applied to a Serum Conferring Protection against Poliomyelitis.

As enlarging the general scope of the work, a test was made of whether exhaustion with red cells would deprive the serum of a monkey recovered from poliomyelitis of its protective power against this disease.[12]

The serum of an immune monkey might conceivably be used in the treatment of human beings after exhaustion of its anti-human elements. For this reason the selective absorption was carried out with human cells, though in the ultimate test of protection monkeys were of

[12] The experiment was rendered possible through the cooperation of Dr. Amoss.

necessity employed. The choice of cells was a poor one, because the anti-human elements in monkey serum are extremely weak[13] and are not readily enhanced by tissue injections. The test of the persistence of antipoliomyelitic elements in serum submitted to selective absorption with human cells is in consequence not a drastic one.

Experiment.—A *Macacus rhesus* monkey recently recovered from poliomyelitis, and with severe residual paralyses, was given intravenously on 3 successive days portions of a mixture of defibrinated human blood and an extract in salt solution of human placenta ground with sand. 10 days after the last injection the animal was bled for serum. This on test showed no hemolysin for human red cells and only weak agglutinins. It was exhausted as follows, according to the usual technique.

Mixture.	Hemagglutination.
2.25 cc. of serum + 0.5 cc. of human red cells, incubated 5 min.;	Massive.
and serum transferred to 0.25 cc. of human red cells, incubated 45 min.;	Moderate.
and serum transferred to 0.25 cc. of human red cells, incubated 45 min.	Faint.

The exhaustion was nearly complete, as shown by tests in which one part of serum in graded dilutions + one part of 5 per cent human red blood cells were mixed in Wright's tubes and examined microscopically after 15 minutes at room temperature.

Agglutination.

Serum.	Serum dilution.						
	0	1/2	1/4	1/8	1/16	1/32	1/64
Untreated	C.	−	−	+	+−	Ft. Tr.	0
Exhausted	+	Tr.					

The test of protective power was carried out by Dr. Amoss, who mixed 2 cc. of the exhausted serum with 0.2 cc. of freshly prepared poliomyelitic virus, and injected the whole, after 2 hours incubation, into the cerebrum of a normal *rhesus* monkey. The animal was one of a considerable number receiving an equal amount of the same virus mixed with various sera, so the experiment was well controlled. Eight monkeys were given mixtures of 0.2 cc. of virus + 2 cc. of

[13] Marshall, H. T., *J. Exp. Med.*, 1901–05, vi, 347.

normal human or monkey serum, and all came down with poliomyelitis after from 5 to 7 days and died. The animal receiving exhausted immune serum mixed with virus remained entirely free from the disease.

The indication from this one experiment is clear, that the principle neutralizing the virus of poliomyelitis persists in immune serum exhausted with red cells. Owing to the difficulty of obtaining immune monkeys the work has not been repeated.

SUMMARY.

Attempts to produce antisera in animals to combat specific infections are usually deferred until the cause of the infection has been isolated and grown in pure culture to furnish antigen. It has seemed to us that the fulfillment of these conditions might in some cases be rendered unnecessary through the use of infected tissue itself as an antigen, combined with selective absorption of the antiserum to rid it of elements injurious to the species furnishing the tissue. In order to test this possibility type experiments have been carried out with immune sera effective against known antigens of three different sorts:

1. Sera resulting from the injection of rabbits and a goat with normal guinea pig tissues and a bacterial hemotoxin, the megatheriolysin described by Todd, which hemolyzes guinea pig cells. The sera possessed strong antitoxins for the megatheriolysin but were fatal to guinea pigs. By the method of selective absorption they were rendered innocuous to these animals and were successfully used to protect them from lethal doses of the megatheriolysin.

2. Anti-rabbit dog sera containing antibodies protective against pneumococcus infection. Such sera, subjected to repeated absorption with rabbit red cells, proved capable of protecting mice from pneumococcus infection in exactly the same degree as the unexhausted serum; that is to say, they protected against 100 times the dose of pneumococci that was fatal with normal dog serum.

3. The serum of a monkey recovered from poliomyelitis and repeatedly injected with human red cells and extract of placental tissue. This serum, after selective absorption with human red cells, protected a monkey against an intracerebral dose of poliomyelitic virus

fatal to eight other monkeys given it with normal monkey or human serum.

The results in these instances, purposely chosen for their simplicity, would seem to indicate for the absorption method some usefulness in the study of immunity to infections of unknown cause. In Part II of our paper the method is applied to one such infection; namely, a sarcoma of the fowl engendered by a filterable agent. A general discussion will be found in connection with this portion of the work.

EXPERIMENTS ON THE PRODUCTION OF SPECIFIC ANTISERA FOR INFECTIONS OF UNKNOWN CAUSE.

II. The Production of a Serum Effective against the Agent Causing a Chicken Sarcoma.

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The work detailed in Part I of this paper has served to demonstrate the theoretical usefulness of exhausted sera to combat infections, but it presents no instance of a serum actually resulting from the direct immunization of animals by injections of infected tissue. Such an instance is highly desirable. It has been furnished through experiments with a transplantable chicken sarcoma,[1] known in our laboratory as Chicken Tumor I, which has a filterable agent as its cause. The exact nature of the filterable agent is unknown, but its general characters would seem to place it with the microorganisms.[2] The tumor is a typical sarcoma, highly malignant, and as a rule rapidly fatal to fowls developing it after an implantation with neoplastic tissue or inoculation with the Berkefeld filtrate of a tumor suspension. Some individuals are primarily insusceptible, and in some the growth develops slowly, and eventually retrogresses. The latter fail ordinarily to develop a sarcoma when reinoculated. Repeated unsuccessful attempts have been made to demonstrate antibodies in the blood of fowls in which a growth has retrogressed, and to render others immune to the tumor by injections with heated or dried neoplastic tissue.[3] The tumor cannot be transmitted to geese, ducks, pigeons, or mammals; but attempts to develop an antiserum by the immunization of such animals have been blocked through failure to obtain the tumor-

[1] Rous, P., *J. Exp. Med.*, 1910, xii, 696; *J. Am. Med. Assn.*, 1911, lvi, 198.

[2] Rous, P., and Murphy, Jas. B., *J. Am. Med. Assn.*, 1912, lviii, 1938.

[3] Rous, P., and Murphy, Jas. B., *J. Exp. Med.*, 1914, xx, 419.

producing agent in culture. The employment in these alien species of the neoplastic tissue itself as an antigen, or a filtrate from such tissue, elicits, of course, anti-chicken elements in the immunized individual.[4] The method of specific absorption to obtain an antiserum here finds a direct application.

Immunization of Animals.—The blood of fowls carrying the chicken tumor often contains during the last few days of life the causative agent of the disease; and in the sarcomatous tissue the agent is regularly present in large quantity. Both blood and tissue could therefore be used in the immunization, which was desirable in order to insure the production of a strong anti-chicken serum. Chickens moribund with the growth were bled to death under aseptic conditions, the blood was citrated, and the tumor tissue itself was ground with sand and suspended in Locke's solution just prior to injection. As the causative agent of the growth will withstand repeated freezing and thawing and retains its activity for a long period at low temperature, the material often was kept in the frozen state for days or weeks prior to use.

The first attempts to obtain an antiserum were made with rabbits. A number of these animals were injected intravenously on 3 successive days with a tumor extract in salt solution, and thereafter intraperitoneally every 6 days with citrated chicken blood and a suspension of tumor tissue. But though the serum soon acquired a high content of chicken hemolysins and hemagglutinins it had not the least neutralizing effect on the tumor-causing agent present in Berkefeld filtrates of suspensions of the sarcoma tissue. For this reason work with rabbits was at length discontinued.

Implanted bits of the chicken sarcoma perish at once in mammals, whereas in ducks and pigeons they grow for some days before retrogressing and may form quite large nodules. It seemed from this fact not improbable that birds would prove more favorable than rabbits as producers of tumor antibodies, owing to what might be considered as a partial susceptibility on their part to the neoplastic disease. For Flexner and his associates[5] have shown that in the case of poliomyelitis an immune serum is obtained only in species susceptible to the infection. Geese were used, therefore, in the further attempts to obtain an antiserum Their immunization was carried out as follows:

Goose A received three intravenous injections on successive days of mixed tumor suspension and citrated blood from fowls moribund of the growth, followed thereafter every 6 or 7 days by intraperitoneal injections of the same material. Goose B was given the same sort of material, but only into the peritoneal cavity. From time to time both birds were bled from a wing vein and the sera compared

[4] Bailey encountered this difficulty in experiments on complement fixation with the serum of pigeons inoculated with the growth (Bailey, C. H., *Med. Rec.*, 1915, lxxxviii, 403).

[5] Personal communication from Dr. Flexner.

in neutralizing properties with those of three normal geese. During a long period of immunization no differences could be noted, but on the contrary a similarity in the five sera so entire as to indicate that the blood of one normal goose is just like that of any other in its effect, or rather lack of effect, on the tumor-producing agent. At length, as will be seen from the experiments now to be cited, an immune principle became demonstrable in the serum of the injected birds.

Experiment 1.—The immunized geese, A and B, were bled for serum 84 and 66 days respectively after immunization was begun, and 9 days after the last injection of tumor material. Goose A had received three intravenous and ten intraperitoneal injections, while Goose B had but nine injections, all intraperitoneal. Two normal geese (a and b) were bled at the same time and to an equal amount; namely, 75 cc. The sera were inactivated as usual, and all were submitted to absorption with similar portions of washed chicken red cells, as follows:

Mixture.	Hemagglutination.	
	Immune sera.	Normal sera.
25 cc. of goose serum + 5.2 cc. of chicken red blood cells incubated 1 hr. and	+	0
serum transferred to 4 cc. of chicken red blood cells, incubated 1 hr.	Tr.	0

Cultures taken after the second absorption proved sterile. Preliminary tests showed that the untreated immune serum failed to hemolyze chicken cells when chicken serum was used as complement, whereas these were rapidly destroyed in the presence of guinea pig complement. Consequently the latter was used in the titrations that follow.

Anti-Chicken Titer of the Sera. Hemolysis.—0.2 cc. of inactivated serum in graded dilutions + 0.2 cc. of 1 in 10 guinea pig complement + 0.2 cc. of 5 per cent guinea pig red cells. Incubation and ice box was for 2 hrs. at 38°C. and readings were made after the tubes had stood in the ice box over night.

Serum.		Serum dilution.								Complement + red cells + 0.2 cc. of salt solution.
		0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	
Untreated immune.	A..	C.	C.	C.	Alm. C.	Alm. C.	+++	Tr.	Ft. Tr.	0
	B..	++++	+++	++	+	+−	Tr.	Ft. Tr.	0	
Untreated normal.	a...	Tr.	Ft. Tr.	0	0	0	0			
	b...	"	" "	Ft. Tr.	0	0	0			
Exhausted sera...		No hemolysis by any.								

The selective absorption had completely deprived the immune sera of their relatively strong hemolysin.

Hemagglutination.—This was read in mixtures similar to the foregoing but containing chicken serum (1 in 10) as complement. None of the tubes showed any hemolysis with this complement, but those containing undiluted immune goose serum exhibited a slight hemagglutination. None of the exhausted sera agglutinated chicken cells in the least.

Precipitation.—The normal sera contained no precipitin, but a weak one was present in the immune sera. It was active against dilutions of chicken serum up to, and including, 1 in 40.

In Vivo Tests of Neutralization.—The exhausted sera only were used in neutralization tests. For this purpose mixtures were made of the sera with a Berkefeld filtrate containing the tumor-producing agent, and these after incubation were injected into fowls. In some early experiments mixtures of the filtrate with isotonic saline or Locke's solution were employed as controls, but it was found that they soon lost their tumor-producing activity when incubated, whereas this was retained in mixtures with normal goose serum, either untreated or exhausted. Consequently in the present experiment, as in others to be detailed, the mixtures with normal sera constitute the controls.

The tumor filtrate was prepared by grinding fresh neoplastic tissue with sand, making a thin suspension in Locke's solution, shaking, centrifuging, and passing the clear fluid through one or another of several Berkefeld filters (N). Several filters were used to ensure an active filtrate, since the tumor-producing agent is held back by many of the finer Berkefeld candles, and all the filtrates were united. Now two mixtures were made with the sera: (1) 15 cc. of each exhausted serum + 7 cc. of filtrate; (2) 7 cc. of each exhausted serum + 2 cc. of filtrate. These were incubated for 2 hours at 38°C. They remained water-clear. 1 cc. of a suspension of sterile diatomaceous earth was added to each, and portions of all were injected into each of a number of chickens. The mixtures with immune sera were injected first so that any possible advantage as regards attenuation of the virus during incubation, or neutralization of it, might lie with the mixtures containing the normal serum. Diatomaceous earth was added because, through the tissue injury it causes, the production of tumors by a filtrate is rendered much more certain.[6]

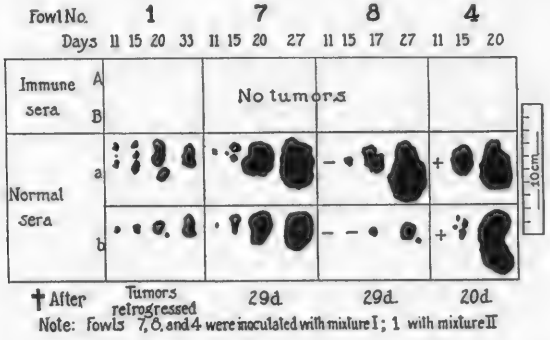
The ten chickens inoculated received 3 cc. of each mixture, into the pectoral muscles and the muscles of the upper wings respectively. Usually the tumor grows fastest and becomes largest in the pectoral muscles, and for this reason the injection site for the mixtures was varied from bird to bird; but in the experiment now under consideration no favoring influence of the pectoral situation

[6] Rous, P., Murphy, Jas. B., and Tytler, W. H., *J. Am. Med. Assn.*, 1912, lviii, 1751.

was to be seen. The growths did not attain a very large size before death ensued from metastases.

Clear-cut findings were obtained, as Text-fig. 1 shows. Only four of the ten fowls developed tumors. In them growths failed to appear where the mixtures of immune sera and filtrate had been injected, whereas at the control sites large ones developed.

Experiment 2.—The same general plan was followed as in the preceding experiment, but the immunized geese had now received two additional intraperitoneal injections. Bleeding for serum was done 121 and 103 days respectively from the time immunization of the birds was started, and 7 days after the last


Fowl No. 1 7 8 4
Days 11 15 20 33 11 15 20 27 11 15 17 27 11 15 20
Immune sera A
B
No tumors
Normal sera a
b
1 cm
† After
Tumors retrogressed
29d.
29d.
20d.
Note: Fowls 7, 8, and 4 were inoculated with mixture I; 1 with mixture II

TEXT-FIG. 1. The tumors in four fowls receiving intramuscular injections of mixtures of tumor filtrate with immune and normal goose sera respectively.

injection. The sera of three normal geese, a, b, and c, were used in control. Selective absorption was carried out as usual.

30 cc. of goose serum + 5.8 cc. of chicken red cells incubated 1 hr. and
serum transferred to 2.9 " " " " " " 1 " "
" " " 2.8 " " " " " " 1 "

Cultures taken after the last absorption proved sterile.

Anti-Chicken Titer of the Sera. Hemolysis.—0.2 cc. of inactivated serum in graded dilutions + 0.2 cc. of 1 in 10 guinea pig complement + 0.2 cc. of 5 per cent chicken red cells.

Serum.		Serum dilution.								
		0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Untreated immune.	A.....	C.	C.	C.	Alm. C.	++	+	Tr.	0(?)	0
	B.....	Alm. C.	Alm. C.	+++	++	+	Ft. Tr.	0	0	0
Untreated normal.	a.....	No hemolysis.								
	b.....	+−	0	0	0	0				
	c.....	+++	++	+	0					
Exhausted sera.....		No hemolysis by any.								

Hemagglutination.—0.2 cc. of inactivated serum in graded dilutions + 0.2 cc. of 5 per cent chicken red cells + 0.2 cc. of salt solution.

Serum.		Serum dilution.				
		0	1/2	1/4	1/8	1/16
Untreated immune.	A.....	+	+	+	Tr.	0
	B.....	++	Tr.	0	0	0

With the exhausted normal and immune sera, as well as the untreated normal sera, no agglutination was obtained.

Precipitation.—There was no precipitin in the normal sera, but one was present in that from both immune geese. It was effective in mixtures of equal parts of the undiluted goose serum with dilutions of chicken serum up to and including 1 in 40 for Goose A and 1 in 20 for Goose B. The titer was little if at all diminished by the absorption with red cells.

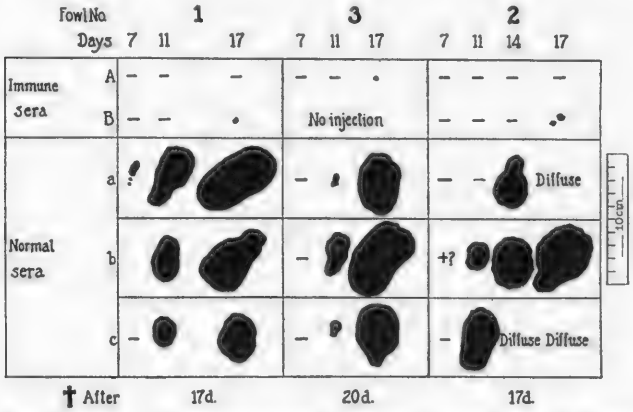
In Vivo Tests of Neutralization.—A Berkefeld filtrate of a tumor extract was prepared by the method already described, and three mixtures were made of it with the exhausted sera, both normal and immune.

Proportion X: 7.5 cc. of serum + 2 cc. of filtrate.
Proportion Y: 12 cc. of serum + 6 cc. of filtrate.
Proportion Z: 7 cc. of serum + 7 cc. of filtrate.

Incubation was for 2 hours at 37°C. No precipitation or clouding occurred. A suspension of diatomaceous earth was now added to each mixture in the amount of one-tenth its volume, and the injection of fowls was forthwith begun. Fifteen fowls were used, and all save four received 3 cc. of each mixture, the site of injection being varied. The four fowls mentioned were not given the mixture con-

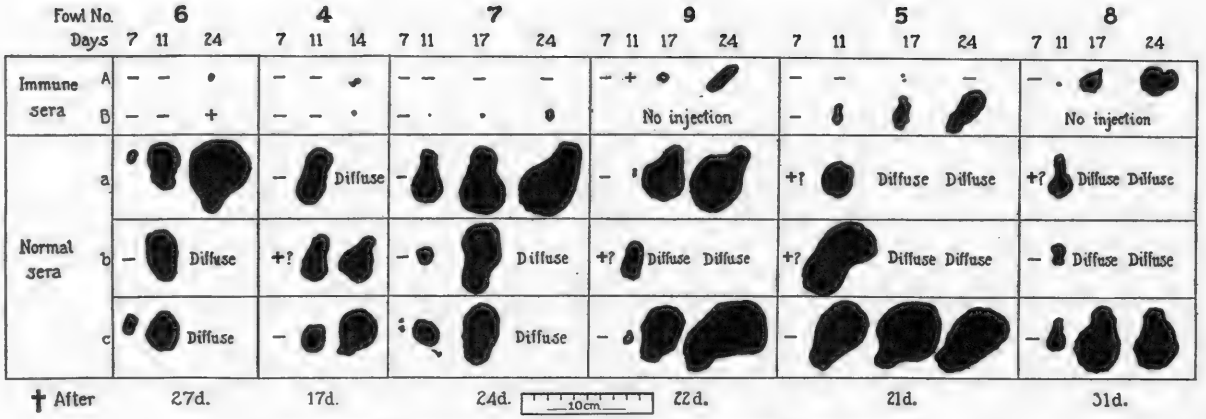
taining the serum of Immune Goose B. The injections were made into the upper wing, upper leg, and pectoral muscles. As Text-figs. 2, 3, and 4 show, large growths rapidly developed where the control mixtures had been placed, whereas none, or only slowly growing ones, were caused by the mixtures containing immune serum.

The neutralizing effect on the tumor-producing agent of the exhausted serum of geese immunized with tumor tissue is clearly shown by these protocols. The agent was especially active in the filtrate used in Experiment 2, as shown by the fact that every one of the fifteen inoculated fowls developed tumors—an occurrence unparalleled in our records. The immune serum completely prevented

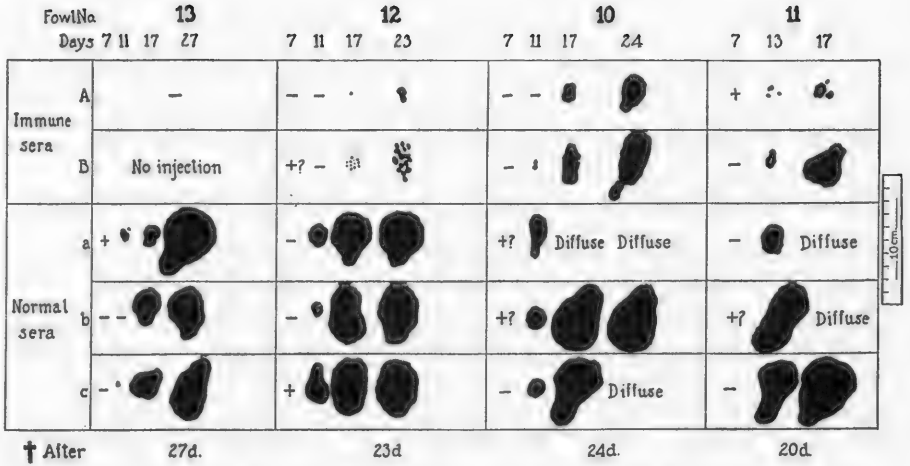


TEXT-FIG. 2. The tumors developing in three fowls receiving mixtures in Proportion X.

tumors at only three injection sites in these fowls, though its protective influence was manifest wherever it had been injected. Very large tumors resulted from all three normal serum mixtures, whence it may be inferred that even the smallest amount of filtrate present in any one, namely that of Proportion X (about 0.66 cc. of filtrate per fowl), contained what might be termed a maximum tumor-producing dose of causative agent. More than twice this amount (1.5 cc. in Proportion Z) yielded tumors that were no larger and grew no more rapidly. The test of the neutralizing power of the immune sera was evidently a severe one in this experiment. In Experiment 1 the filtrate was far less active, as shown by the large proportion of nega-



TEXT-FIG. 3. Tumors in six fowls receiving mixtures in Proportion Y.



TEXT-FIG. 4. Tumors in four fowls receiving mixtures in Proportion Z.

tive fowls (six out of the ten inoculated) and the slow course of the tumors that appeared. Here the neutralization of the tumor-producing agent by the exhausted serum of the immunized geese was complete.

To what is the neutralization referable,—unabsorbed remnants of chicken antibodies? This possibility may be tested by determining whether chicken antibodies as such are able to neutralize the tumor-producing agent. The results with the sera of immunized rabbits gain importance in this connection. For the rabbit sera, while strongly anti-chicken—many times more so than the goose sera—had not the least neutralizing effect on a tumor filtrate.

Experiment 3.—A rabbit was given three intravenous injections on successive days of a saline extract of chicken tumor, followed at 6 day intervals by eight intraperitoneal inoculations of a mixture of tumor suspension and citrated blood from fowls moribund of the growth. 8 days after the last injection the animal was bled to death, and its inactivated serum was compared in neutralizing power with that of a normal rabbit. Selective absorption of both was carried out as usual.

Mixture.	Hemagglutination.
15.5 cc. of rabbit serum + 4 cc. of chicken red blood cells, incubated 1 hr. and serum transferred to 4 cc. of chicken red blood cells, incubated 1 hr.....	Marked. 0

Anti-Chicken Titer of the Sera. Hemolysis.—0.25 cc. of inactivated serum in graded dilutions + 0.25 cc. of 1 in 10 guinea pig complement + 0.25 cc. of chicken red cells.

Immune serum.	Serum dilution.															Guinea pig complement + salt solution + red cells.
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1,024	1/2,048	1/4,096	1/8,192	1/16,384	
Untreated..	C.	C.	C.	C.	C.	C.(?)	Alm. C.	+++	+++	+++	+	±	Tr.	Tr.	Ft. Tr.	0
Exhausted..	+	+	±	±	Tr.	Tr.	Tr.	F. test.	0	0						

Exhaustion was in this instance only approximately complete.

Hemagglutination.—The mixtures were the same as those above except that 0.25 cc. of 0.9 per cent salt solution was substituted for guinea pig complement.

Immune serum.	Serum dilution.									
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Untreated.....	+++	Alm. C.	Alm. C.	C.	C.	++	++	Tr.	—	0
Exhausted....	No agglutination.									

The normal rabbit serum destined to be used in control of the *in vivo* work caused only the slightest hemolysis of chicken cells and no agglutination, when tested prior to its absorption. Thereafter it did not affect the cells at all.

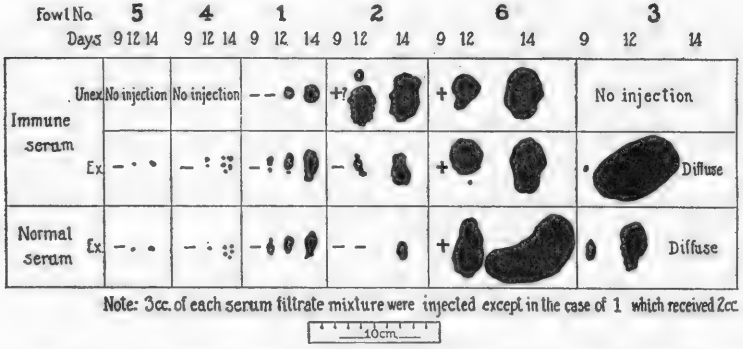
Precipitation.—The normal rabbit serum was entirely inactive, but that of the immunized animal caused precipitation when incubated with equal parts of chicken serum diluted up to and including 1 in 2,560.

In Vivo Tests of Neutralization.—Three serum specimens were used—normal and immune serum, exhausted as above, and untreated immune serum. A Berkefeld filtrate containing the tumor-producing agent was prepared as usual and mixed with the rabbit sera in the proportion of 6 cc. of filtrate to 12 cc. of serum. Incubation at 38°C. was carried on for 2 hours, cultures were taken, portions of a suspension of diatomaceous earth in salt solution were added to each mixture (0.7 cc. for every 20 cc. of mixture), and injections were made of 3 cc. into five fowls and of 2 cc. into a sixth. In the mixtures with immune serum a floccular precipitate had come down which was distributed by shaking prior to the injections. The sites of injection were varied, as usual. The cultures of the injection fluids were negative after 2 days. Tumors developed in all the fowls, as Text-fig. 5 shows.

The test of the neutralizing power of the rabbit sera was in this case not a severe one. For the late appearance and slow growth of the control tumors clearly showed that no excess of tumor-producing agent was present in the mixtures. Yet there is not the slightest indication of any effect upon the agent of the immune serum, even when it had not been exhausted and was very strong in chicken hemolysin, agglutinin, and precipitin. Said serum had exactly the same effect as serum from a normal rabbit, which contained only the weakest antibodies for the chicken. A floccular precipitation occurred in the mixtures of filtrate and immune serum, but so slowly that it can scarcely have afforded to the tumor-producing agent much protec-

tion from other serum antibodies; and only complete protection by it would explain the results in the inoculated fowls.

This experiment would seem to prove that the neutralization of the tumor-producing agent by the serum of immunized geese is not due to antibodies directed against chicken tissue as such. Such antibodies —or at least those elicited in the immunization of rabbits—fail entirely to injure the tumor-producing agent, even when they are very strong. In view of these facts, the conclusion seems justified that the neutralization of the agent causing a chicken tumor by the serum


Fowl No. 5 4 1 2 6 3
Days 9 12 14 9 12 14 9 12 14 9 12 14 9 12 14 9 12 14
Immune serum
Unex. No injection No injection +? No injection
Ex.
Diffuse
Normal serum
Ex.
Diffuse
Note: 3cc. of each serum filtrate mixture were injected except in the case of 1 which received 2cc.
10cm

TEXT-FIG. 5. Tumors arising in six fowls injected with tumor filtrate mixed with normal and immune rabbit serum.

of geese repeatedly injected with the tumor tissue is not the result of the action of antibodies directed against the chicken tissue as such, but is due to others specific for the tumor-producing agent. These are retained by goose serum exhausted with chicken red cells.

DISCUSSION.

The selective absorption of tissue antibodies has been applied thus far to four immune sera of widely different properties (see Part I of this paper), with success in each instance. There is no doubt that by the method sera can be deprived of antibodies immediately injurious to the animal organism while retaining those directed against an infectious agent or its products. Applications of the principle in the treatment of disease at once suggest themselves. But many points

must be determined before any practical therapeutic venture is warranted.

First, the late or latent effects on the animal body of exhausted serum must be closely studied. Serum precipitins are not removed with hemolysins and hemagglutinins during the process of exhaustion with red cells. What then is the effect of a specific precipitin acting *in vivo* on an animal of the species against which it is directed? We have been unable to find in the literature a conclusive answer to this obvious question. The controversy over the relation of precipitation to anaphylaxis has resulted in a multitude of *in vivo* experiments, but these have been carried out almost exclusively by introducing precipitin and precipitinogen into animals to which both are alien, or by injecting a serum precipitinogen into an organism that possesses, or will develop, a precipitin for it. Uhlenhuth and Haendel[7] and Doerr and Moldovan[8] have claimed that anti-guinea pig rabbit serum of high precipitin titer is toxic to guinea pigs when injected intravenously; but these authors made no attempt to absorb from the serum the hemolysins and agglutinins present in it and undoubtedly capable of harmful effects. Their work has not been followed up. We plan to do this.

It seems not unlikely that an antiserum resulting from injections of tissues, especially tissues other than blood, will contain elements of possible harm besides hemolysins, hemagglutinins, and precipitins. Here one is confronted with the problem of the specificity of cytotoxins, so long and indecisively debated. Fortunately we are concerned with a single aspect of this problem; namely, that of whether specific cytotoxins, assuming that they exist for the generality of organs—a large assumption—can be removed from serum by its exhaustion with red corpuscles. For should they not be so removable it may be necessary to exhaust a serum with the same kind of tissue employed in the immunization, a matter of much practical difficulty. Experiments on the point with a specific cytotoxic serum, so called, have been begun.

Theoretically the most important use of exhausted sera lies in the treatment of infections of unknown cause. And with each such in-

[7] Uhlenhuth and Haendel, *Z. Immunitätsforsch., Orig.*, 1910, iv, 761.

[8] Doerr, R., and Moldovan, J., *Z. Immunitätsforsch., Orig.*, 1910, vii, 223.

fection two fundamental points would have of necessity to be determined. They are (1) whether the infected tissue will suffice as a practical antigen, and (2) whether the antibodies useful against the infection or its products will survive the serum's exhaustion of antibodies injurious for tissue. The microorganisms in infected tissue employed as antigen will be in many instances in the highest state of pathogenicity. There are advantages to this, but also drawbacks. If the animals to be immunized are themselves susceptible to the infection much less fresh tissue antigen can be employed than of one attenuated by culture or in another way. The dosage of antigen will also be difficult to regulate. Both these obstacles were encountered in Part I of the present work, during our attempts to immunize dogs by injecting them with the blood of rabbits dying of pneumococcus septicemia. So large a percentage of the dogs died that resort was had at length to an antigen of normal tissues and pneumococcus cultures injected separately. The conditions would be much more favorable to successful immunization in the case of infections only slightly pathogenic to the animals employed for immunization. Here tissue containing the infective agent in most virulent form would have great advantages and not improbably decisive ones in the case of cultivable agents that lose their pathogenicity, and incidentally their usefulness as antigen, when grown *in vitro*. Furthermore, it is conceivable that with an agent in highly virulent form so little of the tissue containing it might in certain instances be required as antigen that the serum's titer in elements injurious for tissue would be slight, and the exhaustion in consequence a relatively simple matter.

Little can at this time be said on the persistence of desirable antibodies in an exhausted serum, further than that our experiments make this seem probable in most instances, as do also the observations of others who have used the method of selective absorption to a different end; namely, to demonstrate the specificity of antibodies.[9] Should it become necessary to exhaust a serum of precipitin by means of precipitation in order to render it harmless *in vivo*, even this, it

[9] A noteworthy demonstration of the possibilities of the method is to be found in the work of Todd, C., and White, R. G., *Proc. Roy. Soc. London, Series B*, 1910, lxxxii, 416. By the selective absorption of induced isohemolysins these authors were enabled to recognize the red corpuscles of individual oxen.

would seem, might be done without, in most instances, removing the antibodies directed against an infectious agent. For Gay and Stone[10] have made many attempts to bring down such elements in a serum precipitate, but without success.

Although the use of exhausted serum in the treatment of infectious diseases is at present but a distant possibility, there lies open a field for its immediate employment. Through the method of absorption much may be learnt regarding serum immunity to animal diseases—as witness the case of the chicken sarcoma,—and to human infections of unknown cause that are transmissible to animals. For the tissues of infected animals will furnish a ready antigen for experimental purposes, while normal individuals of the same species can be used as test objects to determine whether the exhausted sera resulting from immunization possess any protective power. A concrete illustration of such a possibility is afforded by some recent work of Nicolle and Blaizot.[11] These authors state that they have produced an effective antityphus serum in donkeys by injection with the spleens of guinea pigs dying of the disease. The serum is intended for use in human beings, but they find that with it guinea pigs can be cured of typhus, though the serum is so toxic for such animals that it can be given only in small quantities, which hinders the tests. It would have been interesting to deprive the serum of this toxicity by selective absorption with guinea pig cells, with a view to a more striking demonstration of its antityphus power.

SUMMARY.

By the method of selective absorption with tissue, protective serum antibodies have been demonstrated in the case of an infection of unknown cause; namely, a chicken sarcoma transmitted by a filterable agent. Geese were repeatedly injected with the finely ground sarcoma and with blood from fowls moribund of it; and their sera acquired the power to prevent the tumor-producing agent from causing growths. That this was not due to antibodies elicited by the chicken tissue as such was shown by exhaustion of the goose sera with chicken

[10] Gay, F. P., and Stone, R. L., *J. Immunol.*, 1916, i, 83.

[11] Nicolle, C., and Blaizot, L., *Ann. Inst. Pasteur*, 1916, xxx, 446.

red cells, a step which had not the least effect on the tumor-preventing power, and also by experiments with rabbits immunized as were the geese. These animals developed strong chicken antibodies in their sera which failed nevertheless to affect the tumor-producing agent.

Serum immunity to the chicken sarcoma is weak at best; and in the case of some other infections of unknown cause, more striking results may be anticipated from the method of selective absorption. It is even conceivable that by its means sera of therapeutic usefulness may become available. But much remains to be settled as regards the dangers of exhausted sera and the limitations of the method. Fortunately there exists an immediate field for the latter in laboratory studies on the nature of immunity to infections of which the cause has not been recognized.